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SOME ASPECTS OF THE HUMORAL IMMUNE RESPONSE IN THE GOLDFISH *Carassius auratus* AND IN THE TOAD *Bufo marinus*.

by

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, fc acceptance, a thesis entitled SOME ASPECTS OF THE HUMORAL IMMUNE RESPONSE IN THE GOLDFISH *Carassius auratus* AND IN THE TOAD *Bufo marinus*, submitted by LUIGI S. AZZOLINA in partial fulfilment of the requirements for the degree of Master of Science.

ABSTRACT

Antigen-binding and cap formation in the goldfish <u>Carassius auratus</u> have been demonstrated to occur in a range of temperatures between 0^{o} and 37^{o}, using a tritiated flagellar antigen and autoradiography technique.

At temperatures of 10^{o} or less these events take place with higher kinetic rates in cells from fish acclimated at 4^{o} than from fish acclimated at 22^{o}. Fish acclimated at 4^{o} can still produce an immune response, measured in terms of antibody-forming cells (AFC) in the spleen and antibody titers in the serum.

In the goldfish and in the marine toad <u>Bufo marinus</u> the differentiation of the antigen-binding cells (ABC) of the spleen has been followed by velocity sedimentation at unit gravity, which separates cells on the basis of their size. In the spleen of the goldfish the number of ABC seems to diminish one day after immunization, doubles after 2 days and is reduced again one day later.

In toad spleens the ABC are very much reduced 1 and 2 days after stimulation by the antigen, but after 3 days they reappear in numbers that are more than double of those found in normal toads. In both fish and toads these quantitative variations of ABC are associated with changes in cell size.

A secondary response has been obtained in toads primed with two different doses of polymerized flagellin (POL); this was not characterized though by the formation of 2-ME-resistant antibodies.

The injection in toads of the high dose of 100 μg of POL induced a

prolonged response which lasted for more than 34 weeks. Cyclic peak titers of antibodies appeared with a periodicity of 2-3 weeks, which seemed to be controlled by the rate of catabolization of IgM. In fact passively administered IgM had a half-life of 17 days in toads kept at 22^{o} and 12 days in toads kept at 37^{o}.

Passive IgM antibodies could reduce the late appearence of peak titers of actively produced antibody, not the early response.

A faster, but not higher, secondary response was obtained in toads after 3 injections of large doses of sheep erythrocytes, not with small doses.

The first evidence is reported here of the induction in suspensions of toad spleen cells of a primary immune response *in vitro*, using a soluble protein antigen. This response has been studied in terms of AFC and ^{3}H-thymidine uptake, and the basic parameters have been found for the utilization of this system in further investigations.

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ABBREVIATIONS USED IN THIS THESIS

ABC = antigen-binding cell(s)

AFC = antibody-forming cell(s)

Alpha-NPO = 2-(1-naphtyl)-5-phenyloxazole

B- = bone marrow derived

BGG = bovine gamma globulin

BSA = bovine serum albumin

CFA = complete Freund's adjuvant

cpm = counts per minute

DNA = deoxy-ribonucleic acid

DNP = dinitrophenol

EDTA = ethylene-di-amino-tetracetic acid

FCS = fetal calf serum

FITC = fluorescein isothiocyanate

Fc = complement-fixing fraction

G-a-RGG-Fc = goat anti-complement-fixing fraction of rabbit gamma globulin

GG = gamma globulin

H = Heavy

IgG = immunoglobulin of the G class

IgM = immunoglobulin of the M class

IgX = unidentified immunoglobulin

I-IgG = G immunoglobulin from immune serum

I-IgM = M immunoglobulin from immune serum

i.c. = intracardiacally

i.m. = intramuscularly

i.p. = intraperitoneally

KLH = Keyhole Limpet hemocyanin

L = Light

L-15 = Leibovitz' medium

MEM = Eagle's minimal essential medium

MLC = mixed leukocytes culture

N-IgG = G immunoglobulin from normal serum

N-IgM = M immunoglobulin from normal serum

PBS = phosphate-buffered saline

PHA = phytohemagglutinin

POL = polymerized flagellin

PPO = 2,5-diphenyl-oxazole

RBC = red blood cell(s)

RES = reticulo-endothelial system

RFC = rosette-forming cell(s)

RGG = rabbit gamma globulin

RNA = ribonucleic acid

sat. = saturated

S.D. = standard deviation

S.E. = standard error

spp. = species

SRC = sheep red cells

T- = thymus derived

TCA = trichloroacetic acid

u. = international units

w/v = weight per volume

CHAPTER I - THE HUMORAL IMMUNE RESPONSE IN VERTEBRATES

INTRODUCTION

The present study has attempted to analyse some aspects of immuno-regulation in two species of Poikilotherms which appeared particularly suited for experimental purposes. The goldfish *Carassius auratus* is a teleost fish which is adaptable to temperatures ranging from close to 0^{o} to about 30^{o}. The toad *Bufo marinus* is a terrestrial amphibian and is among the first vertebrate species in the phylogenetic order to have lymphoid nodules which resemble the lymph nodes of Homeotherms. In this chapter the current knowledge of the factors affecting immuno-regulation in Homeotherms will be reviewed, before discussing the observations made on Poikilotherms by other investigators, which have constituted the basis for the present work.

THE HUMORAL IMMUNE RESPONSE IN HOMEOTHERMS

The injection of organic substances, either soluble or in suspension, stimulates the body to perform reactions the end-product of which are proteins, known as antibodies (161). These are secreted into the blood and can specifically combine with the injected materials and neutralize them (94). In order for an agent to be antigenic, i.e. to be able to trigger an immune reaction, it must be foreign to the animal, i.e. normally not present in its body.

The ability to produce specific antibodies has been found in all classes of Vertebrates investigated, including the most primitive Cyclostomata (70). The few studies conducted on Invertebrates (25, 34, 38, 78, 139) have shown that these animals have mechanisms other than

antibody production, such as phagocytosis, for neutralizing foreign materials that might enter their body. Invertebrates seem to lack the capability to specifically react to an immunogenic stimulus by multiplication of the reacting cell(s) (22). In contrast, the Vertebrates have evolutionary acquired adaptive responses, consisting of lymphocyte proliferation to form clones of cells each competent for the synthesis and release of antibodies complementary to an antigen (73). According to a current model (40), the immune system may functionally be schematized in the following way. The stem cells produced by the bone marrow proliferate and can either enter the thymus and become thymus-derived (T) antigen-reactive cells, or develop into bone marrow-derived (B) antibody-forming cell precursors. Both cell types then enter the humoral system and circulate through spleen and lymph nodes, where in large part triggering by the antigen occurs.

Upon interaction between T and B cells, with the possible participation of macrophages, B-cells differentiate and proliferate into antibody-forming cells (AFC).

The series of reactions by which the immune system is stimulated, following the introduction of an antigen, may be recapitulated in the following five steps.

1. Recognition of the antigen and related phenomena at the surface of the immunocompetent cells. Spleen and lymph node lymphocytes from normal unstimulated animals can bind antigens to their surface. This has been shown by autoradiography techniques, using radio-iodinated (2, 115) or tritiated (44) antigens, and by immuno-cyto-adherence (122, 166). In B-cells the antigen-binding reaction is mediated by monomeric IgM immunoglobulins (162), present on the surface of the cell membrane.

At appropriate conditions, the receptors may aggregate to a polar region of the cells thus forming what has been called a "cap" (44).

2. Cellular proliferation. Before the antigenic stimulation takes place, no specific AFC are detectable in the animal. They appear in the peripheral lymphoid organs 1-2 days after the injection of the antigen and increase in number logarithmically for 1-2 weeks, mainly as a result of cell division. Cell proliferation does not seem to be the only mechanism by which AFC are recruited. Kinetic studies of AFC populations have pointed out the discrepancy between the generation time of lymphocytes and the doubling time of AFC (23, 54, 55, 81, 125, 136). Therefore the logarithmic growth of AFC seems to be the result of both proliferation and recruitment of cells from AFC precursors (83).

3. Synthesis and secretion of humoral products. Antibody synthesis is a rapid biochemical process. Studies *in vivo* and *in vitro* have shown that labelled amino acids are incorporated into antibody molecules within 20 minutes (85). The light and heavy (L and H) peptide chains composing the immunoglobulin molecules are synthesized on different polyribosomes, assembled in the endoplasmic reticulum and secreted by means of the smooth membrane (8). The first class of antibody to appear in the serum of an immunized animal is the high molecular weight IgM (19S). After a few days a transition occurs from IgM to IgG (7S) production (120). During this phase it is possible to detect few cells secreting both IgM and IgG of the same specificity (119), as well as cells with surface IgM receptors and intracellular IgG (127).

4. Termination of the immune response. The termination of the normal humoral immune response is mainly due to two factors: the cataboliza-tion of the antigen, which causes the removal of the inducing stimulus

(133), and the life-span of the AFC (150). When the antigen is not readily eliminated, the production of antibody can continue for several weeks. This has been shown to accur in responses to poorly catabolized antigens such as E. coli lipopolysaccharide (19), and aggregated forms of serum protein antigens (133). Such antigens cause the cyclic appearance of AFC in the peripheral lymphoid organs.

The four mentioned phases compose the primary response. A subsequent injection of the same antigen induces a secondary response which has its own characteristics.

5. The secondary response. It has been known since antiquity, in relation to pandemic diseases, that survival of the first exposure to antigenic elements such as smallpox, provides immunity against further exposure episodes to the same antigen. Modern studies have contributed to the characterization of the secondary anamnestic response. Features of this response are: a short latent period, increased number of AFC (112) and production of mainly IgG antibody, which has a higher affinity for the antigen than IgM antibody (164).

OVERALL KINETICS IN HOMEOTHERMS

There are several indications that antigen and antibody, among other factors, exert a regulatory function on the humoral immune response.

1. Regulation by antigen. According to the Clonal Selection Theory of antibody formation (21), for each antigen there are in the body few lymphocytes with the potential of specific reactivity. The number of cells in each clone has been estimated to be in the order of one in 50,000 lymphocytes present in the body (117). The antigen affects clonal proliferation and antibody formation in different ways.

Antigens can induce antibody formation when their concentration is above a certain threshold and there is, within limits, a direct relationship between increasing doses of antigen and the resulting titer of antibody. Increasing doses of antigen seem to have a double effect: they increase the amount of antibody produced and, after the primary response, leave in the body increasing numbers of antigen-sensitive "memory cells" (113). Eccessive doses of antigen tend to induce tolerance, defined as "specific unresponsiveness" (49).

2. Regulation by antibody. It has been known for over half a century that excess antibody has a suppressive effect on the immune response (148). Modern studies have attempted to verify the basic concepts of antibody-mediated immunosuppression, according to which antibody is suppressive either by binding with the specific antigen, neutralizing its immunogenicity (peripheral block), or by combining with immunocompetent cells to form receptor-antigen-antibody complexes which have been shown to exert tolerance induction (135).

The regulative effect of antibody appears to be dependent mainly on two factors: its concentration and the immunoglobulin class to which it belongs. The dosage of passively-given antibody effective to suppress active antibody formation seems to depend on the antigen used. In the case of diphteria toxoid, which has 6-8 combining sites for antibody (126), enough rabbit antibody to neutralize one half of the epitopes is sufficient to suppress anti-toxin formation in the guinea pig (156). On the other hand, in the rabbit the response to Keyhole Limpet Hemocyanin (KLH) is not suppressed unless very large excess of antibody (550:1) is present to saturate all the antigenic sites (47). Passive antibodies are effective when given at short intervals before or after

the antigen; increasing intervals between passive immunization and injection of antigen result in decreasing suppression (157).

Under certain conditions, small doses of antibody can have a stimulatory effect rather than a suppressive one. In the rabbit this effect seems to be mediated by complement (157).

A feedback mechanism has been proposed to explain the regulation of the IgM response by IgG antibody, according to which increasing concentrations of circulating 7S antibody would switch off the synthesis of 19S antibody (137). This hypothesis has received some experimental confirmation (62), but it cannot receive universal application. In fact the cyclic response to E. coli lipopolysaccharide is characterized by the synthesis of IgM antibody only (19). In this latter model it has been shown that feedback inhibition could occur within the same class of antibody, since passive specific IgM, given at appropriate times, suppressed the appearance of the cyclic peaks of 19S-AFC (19).

Two research groups have contributed to the understanding of whether suppression by passive antibody acts at the peripheral or at the central level of the immune system (99, 164). Since it was found that the affinity for the antigen of 19S antibody is lower than that of IgG (99), and the affinity of antibody and receptor-sites increases during immunization (164), it follows that the realization of a peripheral or a central antibody effect depends on the state of the active immunization.

Immunosuppression at the peripheral level by passive antibodies can be induced in vivo in the early phase of the response (62). Otherwise it has been obtained in vitro using small amounts of antigen which reacts preferentially with high affinity antibodies (61). In both cases the passive antibodies would compete successfully for the antigen with

the immunocompetent cells, in the first instance because the injected IgM is more avid for the antigen than the freshly immunized cells; in the second case because the small amount of antigen would select the most avid antibodies (112).

In support of the hypothesis of a central effect of passive antibodies, it has been observed that they can suppress the activation of a small clone of AFC-precursors, but are not so effective in suppressing either the expanded clone of AFC or the memory cells, which react normally to antigen-antibody complexes (134). This hypothesis has been further investigated by other authors (61). They have demonstrated that, in the presence of antibodies, the proliferation of AFC in culture follows a slope similar to that of the unsuppressed cells, indicating that the suppression occurred at the level of the potential AFC, thus reducing their number without affecting the quality of the antigenic stimulus. The other hypothesis for a central effect of passive antibodies proposes that these specific molecules prevent cellular interactions required for a normal immune response (135), as will be discussed in the next section.

3. Regulation by cellular interactions. In mice the removal of the thymus at birth results in reduced antibody formation in the responses to sheep erythrocytes (SRC), bovine serum albumen (BSA), influenza virus, diphteria toxoid, Salmonella spp. H antigens, ovalbumen (OA) and T_2 coliphage, while the responses to hemocyanin, Pneumococcus type III capsular polysaccharide and other bacterial and viral antigens remain normal (87, 124). The concept that the normal immune response could be the result of collaboration between cells from different ontogenic origin derives from experiments in which the thymus was reim-

planted in thymectomized animals, either in direct contact with other tissues (106), or in Millipore diffusion chambers (124). The latter experiment showed that a soluble factor was the mediator of the T-cell help. The direct evidence that both T- and B-cells were required for the response to SRC came from one experiment, in which it was shown that lethally irradiated mice required the injection of both thymocytes and bone marrow-cells for immune restoration (27). Further studies utilizing chromosome markers to identify T- and B-cells revealed that the production of antibodies was the function of B-cells and that T-cells, by reacting specifically with the antigen, facilitated their triggering (107, 121).

According to the "antigen focusing" theory, the T-cells, which recognise and are triggered by the carrier part of the antigen, present the haptenic portion to the B-cells (111). This theory does not take into account a further aspect of cellular interaction which concerns the role of macrophages. It has been shown that macrophages have membrane receptors for the complement and, in its presence, they can bind heterologous red cells coated with antibodies (95). Furthermore, B-lymphocytes adhere to macrophages and do so in larger number after being primed with an antigen (140).

The finding that T-cells, upon interaction with the antigen, can release a specific, high-molecular-weight factor which, in the presence of macrophages (58), activates B-cells (59), has lead to the proposition of the "matrix model" for cellular interaction. According to this theory, activated T-cells release an "IgX" which complexes with the antigen and, via the macrophages, triggers AFC-precursors (60).

The recent finding that low concentrations of 2-mercapto-ethanol (2-ME)

can replace in vitro the requirement for macrophages in the response to SRC (24), casts some doubt on the matrix theory. Besides, other functions than just passive presentation of the antigen have been attributed to macrophages. They include the concentration of the antigen to immunogenic levels (160), processing of the antigen to improve and prolong its immunigenicity (159,110), release of RNA associated with the processed antigen (18).

REGULATION AT THE SINGLE-CELL LEVEL

Antigen bound by immunocompetent B-cells of various species has been shown to cause the redistribution of the receptor sites. This phenomenon, called capping, requires active cellular metabolism, which in mammals is optimal at 37^{o}. Therefore mouse cells fail to cap at low temperature (44). This observation is consistent with the fluid mosaic model for the structure of cell membranes (147). According to this, the mobility of the receptors in part depends on the fluidity of the phospholipid bilayer which is determined by the content-ratio between saturated and unsaturated fatty acids.

The significance of antigen binding and receptor redistribution is not yet clear. Cell "suicide" experiments (3), in which lymphocytes that bind ^{125}I-labelled POL lost their ability to respond to the antigen because of radiation damage, suggest that the antigen-binding cells (ABC) include the immunocompetent cells. This hypothesis has been confirmed using the rosette-formation technique (166), and in experiments involving cell separation techniques, from which it is evident that ABC undergo blastogenesis in vivo after antigenic stimulation (93). Furthermore, it has been shown that ABC purified from

primed spleen-cell suspensions by means of a fluorescence-activated cell sorter, give rise to an immune response (84). It appears therefore that ABC form a functionally heterologous population of cells, among which are those committed to produce the humoral response.

PHYLOGENESIS OF THE LYMPHOID SYSTEM

The evolution of the lymphoid tissues in Poikilotherms has been summarized in Fig. 1 (35) and in Table I.

The Cyclostomes are the most primitive Vertebrates and can be considered the starting point in the evolution of the lymphoid organs, bearing in mind that studies on the Protochordates, so far neglected, could bring new information.

Both the pacific hagfish Eptatretus stoutii and the atlantic one Mixina glutinosa lack discrete lymphoid organs, but mononuclear cells are present in the blood and in foci along the gut and in the pronephros (72). These cells have been considered equivalent to the small lymphocytes present in Homeotherms (79). The first evidence of a systematic organization of lymphoid tissues has been observed in the lampreys Petromyzon marinus and P. fluvialis (64). These animals have lymphocytes of different sizes in the blood, in a discrete primitive spleen, in the bone marrow of the protovertebral arch and in foci between the 2nd and 5th pharyngeal pouche: the latter ones have been considered as the first phylogenetic evidence of thymic tissue.

A further step in evolution has been observed in the primitive Elasmobranchs. The adult guitarfish Rhinobatus productus has a discrete encapsulated spleen, which after birth differentiates into white and

Fig. 1

The phylogeny of the lymphomyeloid system in Poikilothermic Vertebrates.

(Adapted from Ref. (35)).

The Thymus and Lymphomyeloid System in Poikilothermic Vertebrates

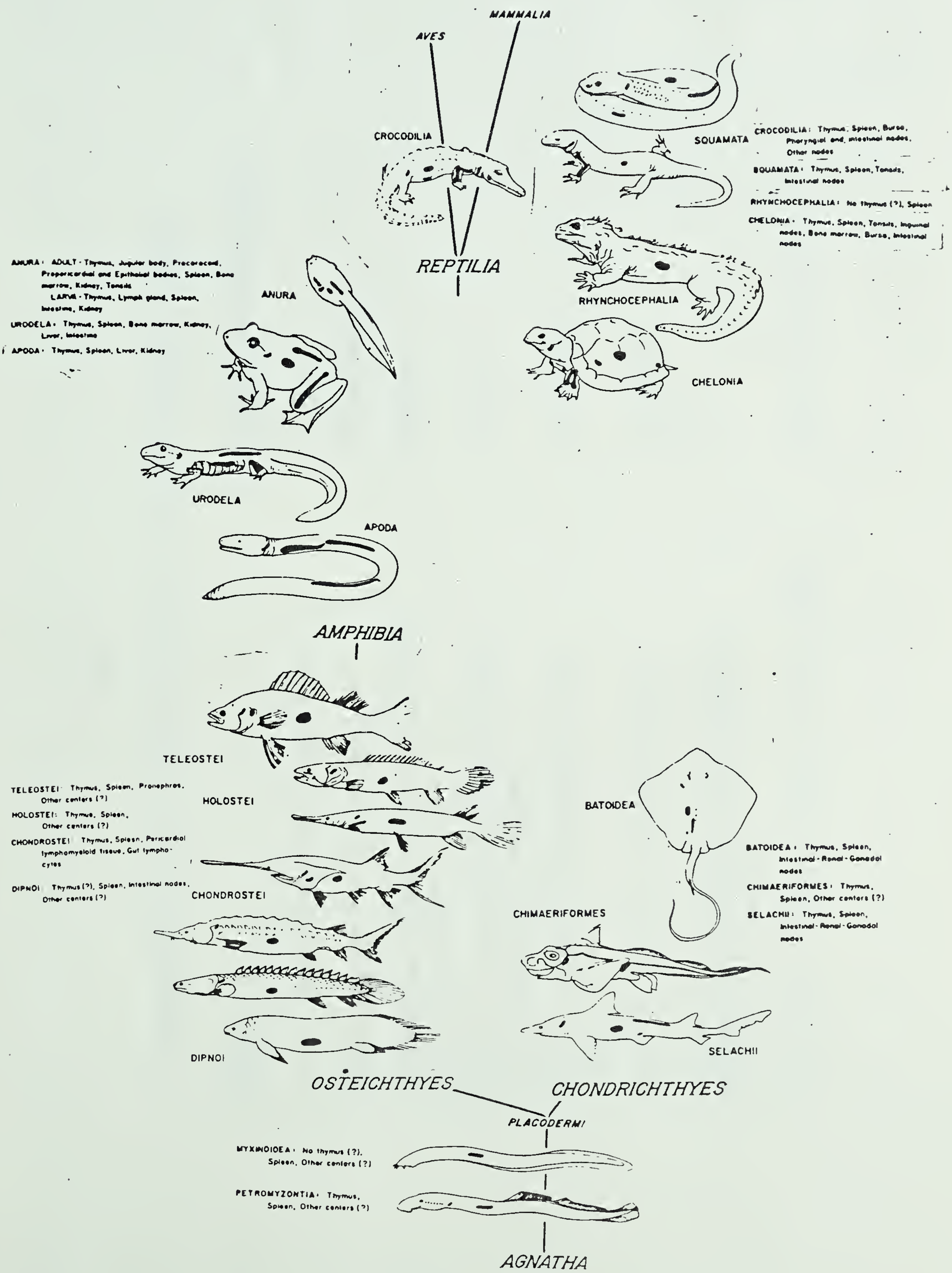

TABLE I

THE IMPORTANT STEPS IN THE EVOLUTION OF THE LYMPHOMYELOID TISSUES

Tissue	Phylogenetic appearence
Foci of mononuclear cells in the gut	Mixinoidea
Spleen, thymic and marrow tissues	Petromyzontia
Encapsulated spleen with red and white pulp, encapsulated thymus	Batoidea
Medulla, cortex and Hassall's bodies in the thymus, plasmacytes	Selachii
Lobes in thymus, lymphocytes in the ileocaecal valve	Chondrostei
Lymphoid tissues in the pronephros	Teleostei
Marrow in the long bones, lymphoid tissues, in the liver and in cervical and popliteal nodules	Anura
Tonsils and lymphoid tissues in the cloaca	Chelonia, Crocodilia

red pulp. In the newborn the thymus is already developed as a discrete encapsulated organ and lymphoid tissues can also be found in the gut, in the gonads and in the renal parenchyma (72).

In the more evolved sharks, like the leopard shark Triakis semifasciata and the nurse shark Ginglimostoma cirratum, the thymus is differentiated into cortex and medulla, and there are epithelial accumulations resembling Hassall's bodies. In these animals the thymus does not seem to involute with age, as it does in the more evolved Vertebrates (72). In these species plasmablasts and other plasmacytes are present in the spleen, especially after antigenic stimulation (56).

The evolution from the cartilagenous to the bony fish has led to definite modifications in the thymus and the spleen, which acquired a histological organization comparable with that of Mammals. In the Chondrostean paddlefish Polyodon spatula the thymus is organized into lobes with primitive Hassall's corpuscles. The spleen is well divided into white and red pulp, containing numerous cells identical to those found in Mammals. Aggregations of lymphocytes can be found in the region of the ileocaecal valve and in hemopoietic tissues around the heart (72). In the highest fish, the Teleosts, lymphocytic foci are also found in the pronephros, a characteristic which is later transmitted to the Amphibia.

The passage from the aquatic to the terrestrial environment, requiring among other adaptations the development of long bones for deambulation, brought along a more complete diversification of the lymphoid system to include the bone marrow of the femur, the liver and secondary lymphoid nodules in the neck region. The Apoda maintain a fish-like lymphoid system, with the only exception that the liver also

functions as a lymphomyeloid organ. The bone marrow is already evident in the Urodeles, but in the Anurans it is more active and contains both lymphocytes and plasma cells (37). Lymphoid nodules that can be considered as primitive lymph nodes have been described in the marine toad Bufo marinus to occur along the jugular vessels (41, 88). They can also be found in the popliteal spaces after intense antigenic stimulation of the collateral hind foot pad (71). Lymph glands composed of macrophages and lymphocytes have been observed in the larvae of the bullfrog Rana catesbiana (33). The reticulo-endothelial system (RES) has been studied with positive results in the clawed toad Xenopus laevis (153). The presence of pharyngeal tonsils in the Anurans is controversial, but it has been confirmed in two Families of Reptiles, the Crocodiles and the Chelonia. In these two groups there is also the first evidence of a lymphoid organ in the cloacal region, which has been considered homologous to the bursa of Fabricius found in birds , (72, 143).

THE HUMORAL IMMUNE RESPONSE IN POIKILOTHERMS

Investigations on the functional aspects of the immune system in Poikilotherms have been mainly concentrated on the responsiveness to tissue and soluble antigens and on the type of specific immunoglobulins obtained.

From the most recent literature it is evident that even the most primitive cyclostome, the hagfish, is capable of responding to the injection of antigens with the production and secretion of specific antibodies which have the characteristics of immunoglobulins (98, 152). Therefore the synthesis of L and H polypeptide chains, their union to form specific antigen-binding sites and their polymerization into mo-

lecules containing two or more antigen-binding sites, seem to be characteristics present throughout the whole vertebrate phylum.

The different phases of the immune response have been only occasionally studied in Poikilotherms.

1. Recognition of the antigen. Few observations have been gathered on the initial steps of immuno-induction in Poikilotherms, mostly in Anurans.

In the rainbow trout *Salmo gardneri* few rosette-forming cells (RFC) can be found in the spleen and pronephros before immunization with SRC (26). Such RFC have also been observed in the Anurans *Alythes obstetricans* (51), *X. laevis* (89) and *B. marinus* (31). Within this group *X. laevis* seems to have the most primitive antigen-trapping system. In these animals injected intraperitoneally (i.p.) or intramuscularly (i.m.) with BSA the antigen persists in the serum for as long as 26 weeks, and specific antibodies are not detectable (80). The injection of human gamma globulin (GG) in complete Freund's adjuvant (CFA) stimulates a good response but the antigen is still persisting in the serum after 5 weeks (100). On the other hand, by injections of carbon particles and of human blood, a well developed RES has been observed in *X. laevis*. In the larvae pericardial and peritoneal macrophages are responsable for clearing i.p.-injected carbon. In the adult the carbon-ingesting macrophages are found also in the pro- and mesonephros. Foreign blood particles are removed by spleen and liver cells in both larvae and adults, and by the kidney in adults (153). In the more evolved *B. marinus*, using colloidal carbon and ^{125}I-labelled *Salmonella* flagellar antigen, it has been shown that the localization of foreign particles occurs mainly in both reticular and lymphoid cells of the

jugular bodies, in the white pulp of the spleen and in the kidney (41). While in B. marinus the distribution of the antigen in the lymphoid organs is random, in the lymph glands of R. catesbiana a pattern reminiscent of primitive germinal centers has been observed after antigenic stimulation (35).

In conclusion, antigen recognition and trapping in the lower Vertebrates seem to depent mainly on the activity of the phagocytic cells, which do not eliminate certain antigens, such as soluble serum proteins, unless presented with adjuvants. In the more evolved Anurans the presence of secondary lymphoid organs such as jugular bodies and lymph glands improves the efficiency of reactivity.

2. Cell proliferation. In lampreys injected i.m. with BSA in CFA there is a marked increase in the number of large mononuclear cells in the marrow of the protovertebral arch. That this is due to proliferation has been demonstrated by ^{3}H-Thymidine uptake 11 days after immunization (63). X. laevis toads react to the injection of SRC and to human GG with a significant enlargement of the spleen, in which the areas at the periphery and the white pulp are most affected. This process is accompanied by the increase in number of pyroninophilic cells (155).

Four days after immunization with polymerized flagellin (POL), more than 70% of AFC from B. marinus spleen take up ^{3}H-Thymidine, a finding which indicates that the increase in the number of AFC is largely due to cell proliferation (43).

Cell proliferation has also been demonstrated in vitro. X. laevis (163) and B. marinus (69) spleen cells react in mixed lymphocyte cultures (MLC) by uptake of ^{3}H-Thymidine with kinetics compatible with those observed in mammalian cells.

3. Secretion of antibodies. Specific immunoglobulins have been found not only in the serum but also in the mucus of fishes. In the gar *Lepisosteus platyrhincus* it has been observed that the titer of anti-SRC antibodies in the mucus secreted by the skin increases by 3 $\log_2$ after immunization, and is sensitive to treatment with 2-ME (17). This is in agreement with the general finding that the humoral response of Poikilotherms is restricted to the production of immunoglobulins of the IgM class. In the hagfish the immunoglobulins obtained after immunization with SRC and KLH belong to the IgM class and have a sedimentation coefficient of 23.8S. A 7S protein which does not contain antibodies but is antigenically similar to IgM was also found (98).

Lampreys immunized with f_2 bacteriophages produce two types of antibodies, both with IgM characteristics, but sedimenting as 14S and 6.6S proteins (104).

Even though it has been shown that the serum of normal sharks contains up to four antigenically different immunoglobulins (68), in the immunized smooth dogfish *Mustelus canis*, tested in the first month after the injection of antigen, the antibody activity has been found in the 19S class only (102). In late antisera from the lemon shark *Negaprion brevirostris* low-molecular-weight antibodies of about 7S can also be found (29). Yet by different parameters, such as molecular weight and electrophoretic mobility of the H chains, and hexose content, both 19S and 7S antibodies have been shown to belong to the same class (30).

15S and 7S anti-DNP antibodies have been obtained from the bowfin *Amia calva* (15), but not from another species of Holosteans, the gar *L. platyrhincus*, immunized with diphteria toxoid, BSA or *S. typhosa* H antigen; all these induced high-molecular-weight antibody only, even after one

year of immunization (16). Slow sedimenting (7S) antibodies have been obtained in the goldfish C. auratus repeatedly immunized for 3-5 months with bacteriophage ØX 174. These 7S antibodies, like the 19S ones obtained in the first month after immunization, were almost completely sensitive to 2-ME treatment (158).

High-molecular-weight antibodies resistant to 2-ME have been obtained in the secondary response of the perch Perca fluviatilis to human GG. These antibodies have an S value of 14.5 and, according to their electrophoretic mobility, have been classified as $Beta_1$ globulins (5).

Amphibia may represent the first Class of Vertebrates in which antibodies closely related to mammalian IgG have appeared. R. catesbiana immunized with f_2 phages synthesizes 19S and 7S antibodies. The latter type has been considered similar to IgG by molecular weight of the H chains, carbohydrate content, amino acids content and electrophoretic mobility; however this antibody was still sensitive to 2-ME (103). The same results have been obtained in B. marinus immunized with T_2 phages, but not with S. typhosa H antigens, which induce the formation of 19S antibodies only (97). The low -molecular-weight and 2-ME-sensitive antibodies found in the apodan Ophisaurus apodus have been classified as $Beta_1$ globulins (5).

Immunoglobulins with complete IgG characteristics have been found in the more evolved Reptiles. The chelonid Testudo hermanni responds to the injection of pig serum with an almost mammalian-type sequence of antibody production, in which low-molecular-weight and 2-ME-resistant gamma globulin antibodies appear in the late response (Table II) (5). Similar results have also been obtained in the crocodilian Alligator mississippiensis, which has been shown to possess two antigenically

TABLE II

Characteristics of the antibodies produced by the turtle *Testudo hermanni* (Gmelin).

	Antibodies			
	$early_1$	$early_2$	$late_1$	$late_2$
Molecular type	high-molec. wt.	low-molec. wt.	low-molec. wt.	extreme low-molec. wt.
ME-stability	sensitive	sensitive	stable	sensitive
Electrophoretic mobility	$Beta_2$ glob.	Gamma glob.	Gamma glob.	?
$s_c^{20\ C}$ value	18	7	7	4,5

Antigen: Pig normal serum. (Adapted from Ref. (5)).

distinct L chains, perhaps comparable to the Kappa and Lambda chains of Mammals (138).

4. Duration of the response. Good immunogens tend to induce a prolonged response in some Poikilotherms, the duration of which can extend for over one year. In the lamprey P. marinus the response to Brucella abortus has been followed for 32 days, when antibodies were still present in the serum (64). In Elasmobranchs the response to PR8 virus peaks at 30 days, but antibodies are still detectable in the serum 100 days after immunization (146). In Holosteans and Teleosts immunized with BSA the primary response lasts for at least 88 days (28), and titers of up to 1:320,000 have been found in the serum of P. fluviatilis 150 days after immunization with human GG (5).

Long term persistance of antibody formation has also been reported in Amphibians and Reptiles. In X. laevis immunized with human GG in CFA low titers of antibody are still present 8 months after immunization (100). The response to POL in the marine toad B. marinus has been reported to last for at least 65 days (43).In the turtle Testudo hermanni the humoral response to pig serum can continue for months (5).

The long-term formation of antibody observed in most species of Poikilotherms seems to be due to two reasons: the long life of the AFC, which contrasts with the short life of the AFC found in Mammals (150), and the persistence of the antigen in the body. In B. marinus it has been found that the logatithmic increase in the number of AFC during the first ten days after immunization with POL is mostly due to cell divisions. Then, while the total number of AFC declines slowly, the number of AFC labelled with ^{3}H-Thymidine diminishes more rapidly, indicating that most AFC have survived from the early proliferation (91). The persistence of the

antigen in the serum of X. laevis immunized with human GG in CFA has been detected 5 weeks after immunization, at a time when the humoral response was already in progress (100). It is therefore possible that Poikilotherms, in regard to some antigens, have a poor catabolic mechanism which cannot readily eliminate the antigenicity of the injected material, especially above a certain dose-treshold.

5. The secondary response. The first species in the phylogenetic order in which an anamnestic response has been observed is the lamprey P. marinus. In this animal not only second-set homograft rejection and delayed allergic responses to tuberculin have been demonstrated, but also an accelerated and increased production of specific antibodies after a booster injection of B. abortus antigens (64).

In higher species the studies on the presence of immunological memory and the capacity to produce a secondary response have given results which sometimes appear in conflict with each other.

According to some Authors, the horned shark Heterodontus francisci displays immunological memory because it responds with high titers to a second injection of hemocyanin, T_2 phages or killed Brucella cells. In those experiments no antibodies were detected after the priming injection, although the antigens were cleared from the circulation within the first two weeks (65). Another group of investigators has found that the lemon shark N. brevirostris immunized with multiple injections of PR8 virus during a period of 4 weeks produces a strong primary response which peaks at 30 days and then plateaus for about 5 months. A booster injection of the antigen at this time raises the antibody titer to the same level as the primary peak. On the other hand, a single priming injection of concentrated PR8 virus induces a weak primary response; re-

peating the immunization 30 days after the first one, the titer increases drammatically, but reaches the same levels obtained with multiple priming injections (146).

The same kinetics have been observed in fishes. The holostean gar L. platyrhincus immunized with high doses of BSA reacts with a strong primary response which lasts for at least 70 days. A second injection given at this time lowers the existing titer of antibodies (28). The teleost margate Haemilon album immunized with a moderate dose of BSA responds in a primary fashion which can become secondary after a subsequent injection of antigen. On the other hand, when the same priming dose of antigen is given in CFA, the primary response is much stronger, and subsequent injections raise the antibody titer by just one or two $\log_4$. Similar effects were obtained by giving multiple injections of the same dose of BSA in the first 20 days of immunization (28).

The amphibian B. marinus does not produce a secondary response when primed with high doses of Salmonella antigens (41, 57), but shows memory against T_2 phages (57). In Reptiles the turtle Chrysemis picta fails to respond in a secondary fashion when the second injection of KLH is given during the exponential phase of the primary response (74). In contrast, the lizard O. apodus gives secondary and tertiary responses to pig serum when the second and third injections are made during the declining phase of the preceding response (5).

These results suggest that strong immunogenic doses can stimulate such a high primary response that a secondary one cannot be demonstrated unless one waits long enough for the response to the first immunization to fade out.

Another reason for what could appear as a poor secondary response

is natural immunization. In fact it has been found in the gar L. platyrhincus that repeated injections of diphteria toxoid or BSA can stimulate a good secondary and tertiary response. On the other hand, injections of S. typhosa H antigen did not enhance antibody synthesis, the apparent reason being that all pre-immunization sera contained specific natural antibodies with titers ranging from 1:16 to 1:512. Therefore the adaptive response observed as a primary one was actually an anamnestic response by animals naturally immunized (16).

REGULATORY FACTORS IN POIKILOTHERMS

1. Effect of antigen. Besides the effects on the secondary response mentioned in the preceding section, the type and dose of antigen can variously influence the primary response.

In the toad X. laevis the humoral response to cellular antigens such as SRC takes 28 days to reach its peak, while the optimal response to human GG is not obtained before 56 days (155), and BSA seems to be non-immunogenic (80). Similarly, in the toad B. marinus the titer of antibodies against SRC is optimal after 7 days (31), while the response to POL is best after 2 weeks (43). In this species increases in dosage of BSA over the optimal immunogenic one result in a decreasing response, until specific unresponsiveness is achieved (105).

2. Effect of temperature. Since the physiological functions of Poikilotherms are more dependent on the environmental conditions than those of Homeotherms, different results could be obtained from the same animal tested at different temperatures. Studies on the hagfish kept at 15^{o} failed to show a humoral response to BSA, KLH and bacterial antigens (64), but a positive response was obtained when the animals were kept at 18^{o} (152).

The goldfish C. auratus has been shown to resist bacterial infection better at 23^{o} than at lower temperatures (12). The same animal can reject scale homografts at 10^{o}, but the rate of rejection increases with the experimental temperature, up to 32^{o} (77). Eels injected with Vibrio spp. form antibodies at 18^{o}, but do not so at 8^{o} (123). The response to POL in the marine toad B. marinus is faster at 37^{o} than at 22^{o}; the peak number of AFC is reached in 7 days at 37^{o} and in 14 days at 22^{o}, but the two peaks are not different. On the other hand, the peak titer of serum antibodies is reached at the same time (14 days), but the titer obtained at 37^{o} is significantly higher (43).In the same animals it has been found that the number of RFC against horse erythrocytes is dependent on the dose of the antigen, not on the temperature, within the range of 22^{o} - 37^{o}. By contrast the synthesis of antibody is dependent on both the temperature and the dose of antigen (31).

In the frog R. temporaria the titer of serum antibodies against Pseudomonas fluorescens is higher in animals kept at 20^{o} than in those kept at 8^{o}. From these and other experiments the author concluded that antibody secretion is inhibited at temperatures below 12^{o} (13).

This is not always true, as shown by the following data. The sablefish Anaplopoma fimbria, which lives in the cold waters of the North Pacific, can produce specific agglutinins at 5-8^{o} (131). Species of Salmonids produce higher titers of isoimmune hemagglutinins at 5-10^{o} than at higher temperatures (130). The gar L. platyrhincus and the bowfin A. calva produce antibodies which react best at temperatures around 4^{o} (14, 15). Therefore it is evident that this type of studies require a good knowledge of the animal used, since eurythermic species can tolerate and adapt to a wider range of temperatures than stenothermic ones.

3. Effect of nutrition. Altough it is known that some species do not feed well in captivity, not much attention has been given to this factor which, at least in one species, the marine toad B. marinus, has been shown to affect the physical characteristics of lymphocytes and cause a delay in the AFC response to POL (92).

SUMMARY OF THE PRESENT WORK

Poikilotherms and those homeotherms which exhibit adaptive hypothermy (hibernation) should be able to react immunologically even when their body temperature approaches 0^{o}, in order to survive their exposure to pathogenic agents.

A widely discussed phenomenon observed on the membrane of mammalian lymphocytes is the redistribution of immunoglobulin receptors by specific ligands such as antigens (44). This "capping" process has been demonstrated to depend on the metabolic activity, which in mammals is optimal at 37^{o}.

The adaptability of the goldfish to a wide range of temperatures made it possible to investigate the capacity of its lymphocytes to demonstrate receptor redistribution, and to assess its state of immunocompetence, at different temperatures. As shown in the next chapter, the capping phenomenon can be observed on goldfish lymphocytes at different temperatures within a range of 0^{o}-37^{o}. Prior acclimatization at 4^{o} improves the rate of cap formation. Furthermore, fish acclimated at 4^{o} maintain their capacity to produce a primary immune response, even though at a lower rate than animals acclimated at 22^{o}.

The study of the early phase of immune induction in goldfish and

marine toads is reported in chapter III. Spleen ABC from fish and toads have been characterized by velocity sedimentation at unit gravity, one, 2 and 3 days after in vivo stimulation. Two main observations have been made, a) the population of ABC changes in size between day 0 and 3 of the immune response; b) the total number of ABC/10^6 increases in the early phase of the immune response.

Chapter IV reports the analysis of the kinetics of antibody formation in vivo in the primary and secondary response of the marine toad immunized with POL or SRC. In these experiments it has been found that small and moderate doses of POL prime the animals, which later respond in an anamnestic mode to a second equal dose of antigen. High doses of POL cause a cyclic appearance of peak titers of antibodies, the periodicity of which seems to be regulated by the level of immunoglobulins in the serum. This is concluded from the rate of catabolization of passively administered ^{125}I-IgM. Both the primary and the secondary responses to SRC depend also on the dose of antigen, larger doses being more effective.

Chapter V presents the first evidence of in vitro immunization of Amphibian single cell suspensions with a soluble protein antigen. The response to different doses of POL has been measured in terms of AFC, which appear in largest number between day 5 and 6 of culture and again at day 15. ^{3}H-Thymidine uptake has been found to peak at day 4 of the in vitro response.

The results of the present study will be discussed in chapter VI.

CHAPTER II - ANTIGEN RECOGNITION, CAPPING AND HUMORAL RESPONSE BY THE GOLDFISH *Carassius auratus* ACCLIMATED AT 4° AND 22°

MATERIALS AND METHODS

Animals. Adult goldfish *C. auratus* 5-6" long were supplied by commercial sources. The fishes were kept in glass tanks in dechlorinized and aerated fresh water which was changed every week. They were fed daily with Goldfish Food (Wardley, Long Island, N.Y.). All fish were acclimatized either at 4° or 22° at a rate of 2°/day. Attempts to accelerate the rate of acclimatization resulted in death of the animals. They were kept at either temperature for 2 weeks before being used. Male CBA/J mice bred at the Ellerslie Animal Farms, University of Alberta, 60 to 90 days old were also used.

Cell suspensions. The fishes were killed by cerebral concussion and the spleens were removed aseptically. A single-cell suspension was obtained by passing the minced organs through a fine-mesh stainless steel sieve. The cells were suspended in Leibovitz (L-15) medium (GIBCO, Oakland, Calif.) and washed once before counting in a hemocytometer. Cell viability was determined by eosin dye exclusion (75). The average yield was 65×10^6 viable leukocytes/spleen of fish acclimated at 22° and 35×10^6 viable leukocytes/spleen of fish acclimated at 4°.

Mice were killed by cervical dislocation and their spleens were placed in phosphate-buffered saline (PBS) (50) containing 5% of fetal calf serum (FCS). A single-cell suspension was obtained as for the fish, and the cell concentration was adjusted to 50×10^6 viable leukocytes/ml.

Preparation of the antigen. Purified polymerized flagellin (POL) from *S. adelaide* (strain SW 1338, H antigen fg, O antigen 35) was prepared

according to a method described by others (4). Radiolabelled POL (^{3}H-POL) was prepared by biosynthetical incorporation of tritiated L-Leucine (6 Ci/m.mol., Schwarz-Mann, Orangeburg, N.Y.), following the procedure devised in our laboratory (44).

Antigen-binding assay. Aliquots of 20 x 10^6 viable fish leukocytes in 1.0 ml of L-15 medium were incubated at 0^o for 2 hrs with 250 ng of ^{3}H-POL in plastic Falcon tubes. Then different aliquots of cells were further incubated at 0^o, 10^o, 22^o and 37^o for 1, 2, 3 and 6 hrs. After the last incubation the cells were centrifuged in the cold at 330 g for 10 min, washed twice in L-15 medium and then spun through a 25-100% gradient of FCS in L-15 medium. The pellet was resuspended in FCS, smeared on gelatin-coated slides and fixed in methanol-acetic acid-water (89:1:10) for 30 min.

Autoradiography. The slides were dipped in Kodak NTB-2 photographic emulsion, dried and exposed for 21-30 days at 4^o. Then they were developed with Kodak D-1a, fixed and stained with Gimsa stain.

Screening. At least 5 x 10^5 fish leukocytes were screened for the presence of labelled cells at each experimental point. Cells which had at least 10 grains above background were considered positively labelled. Caps were scored when the grains were congregated in a continuous polar region of the cell.

Fluoroscein-labelling of goat-anti-Fc of rabbit GG IgG. The conjugation of goat IgG anti-Fc of rabbit GG (G-a-RGG-Fc) with fluorescein was done according to the following procedure (132). Three ml of a 10 mg/ml solution of a purified IgG fraction of G-a-RGG-Fc$_{(i)}$ in 0.9% NaCl contai-

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(i) - kindly provided by Dr. C. Shiozawa of our laboratory

ning 0.05 M Na-carbonate-bicarbonate buffer (pH 8.6) were mixed by hand with 7.0 mg of Fluorescein-iso-thiocyanate (FITC) on Celite (10%) (Calbiochem, La Jolla, Calif.). The mixture was then centrifuged at 600 g for 3 min, and the supernatant dialysed twice overnight against 2 l. of 0.9% NaCl. The efficiency of the conjugation was tested by precipitation with RGG.

Assay for cap-formation on mouse cells. The double-layer method (31) was used for this assay. 2 μl of anti-mouse-IgM RGG (10 mg/ml)$_{(i)}$ were added to 10 x 10^6 mouse spleen cells in 0.2 ml of PBS-10% FCS and incubated for 30 min in ice. After the cells had been washed 3x and resuspended in 0.2 ml of PBS-10% FCS, 20 μl of FITC-G-a-RGG-fc were added. After incubation for 30 min at 0^o, the cells were washed 3x at 4^o, resuspended in 0.2 ml of PBS-10% FCS and incubated for 30 min at 0^o, 12^o, 14^o, 16^o, 18^o, 20^o, 22^o, 25^o, 28^o, 30^o, 33^o and 37^o. Finally the cells were cooled in ice, smeared and examined in a Zeiss microscope adapted with U.V. light. 100 or more fluorescent cells were screened and the percentages of caps calculated. For each experimental point individual cell suspensions from 3 or more spleens were tested to calculate the mean percent of cap formation and the standard deviation at each temperature.

Assay for antibody-forming cells. After acclimatization, groups of 6 fishes were injected i.p. with 10 μg of POL. Similar groups were kept as unimmunized controls. At weekly intervals they were sacrificed, the spleens were removed and separate cell suspensions for each animal were prepared as described above. The cells were washed in L-15 medium and

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(i) - kindly provided by Dr. C. Shiozawa of our laboratory

assayed for the number of AFC using the adherence colony method (39).

Test for humoral antibodies. After sacrifice each fish was bled from the heart with a pasteur pipette. The blood was collected individually in Beckman microcentrifuge plastic tubes and incubated at 22° for 30 min and at 4° for 1 hr. The sera were separated by centrifugation, incubated at 56° for 30 min and tested the same day for bacteria-immobilizing antibodies (7), against *S. derby* bacteria (strain SW 721, H antigen Fg, O antigen 1, 4, 12) which share the H but not the O antigen with *S. adelaide* strain SW 1338 from which the immunogen POL was made.

RESULTS

Kinetics of antigen-binding cells in goldfish. The cell surface dynamics that promote antigen binding on murine cells could be demonstrated also on fish lymphocytes. The number of ABC in the goldfish spleen varied between 3 and 86/10^6 white cells, depending on the temperature of acclimation of the fish and on the temperature and duration of incubation of the cells (Table III, Fig. 2). The extreme values were observed in suspensions incubated at temperatures extreme for this fish. At temperatures equal or close to those of acclimation the number of ABC was comparable with the values obtained with mouse cells at 37° using the same antigen (Table IV). At 0° and 10° the number of ABC was higher in the preparations from fish acclimated at 4°, but the difference was not statistically significant. At 22° and at 37° the spleen cells from warm-acclimated fish contained peak numbers of ABC after 2 and 3 hrs respectively, which were followed by a drop to low numbers.

At 37° the cell suspensions from both warm and cold acclimated fishes showed evidence of cell damage, in terms of green coloration and turbi-

TABLE III

RATE OF ANTIGEN BINDING AND CAP FORMATION IN FISH ACCLIMATED AT 22° AND 4°

Temperature of incubation	Hours of incubation	No. of labelled cells/10^6		% caps		No. of grains (average $\pm$ S.D.)	
		22°	4°	22°	4°	22°	4°
0°	1	3	24	50.0	33.3	34.0(16)	41.3(31)
	2	25	25	72.7	25.0	39.0(12)	81.7(27)
	3	11	21	23.0	77.0	64.3(22)	34.5(26)
	6	8	18	36.8	92.0	55.4(26)	43.0(28)
10°	1	17	42	36.8	93.7	50.5(19)	35.6(12)
	2	32	38	46.0	77.0	65.0(27)	41.0(19)
	3	15	19	29.1	80.0	62.1(23)	32.5(15)
	6	23	44	26.8	81.0	56.7(26)	27.5(15)
22°	1	15	21	36.8	55.0	59.0(24)	25.2(13)
	2	68	13	46.6	100.0	61.0(20)	19.0(4)
	3	5	36	57.0	55.0	43.0(9)	34.6(27)
	6	17	32	23.0	75.0	64.7(18)	36.3(22)
37°	1	20	16	81.0	57.8	23.0(6)	26.0(14)
	2	35	8	71.4	25.0	21.7(8)	21.0(10)
	3	86	11	69.2	42.8	19.8(7)	25.0(17)
	6	7	10	100.0	60.0	17.5(5)	26.6(15)

The cells were incubated with 250.0 ng of ^{3}H-POL for 2 hrs at 0° and then for 1-6 hrs at the temperatures shown.

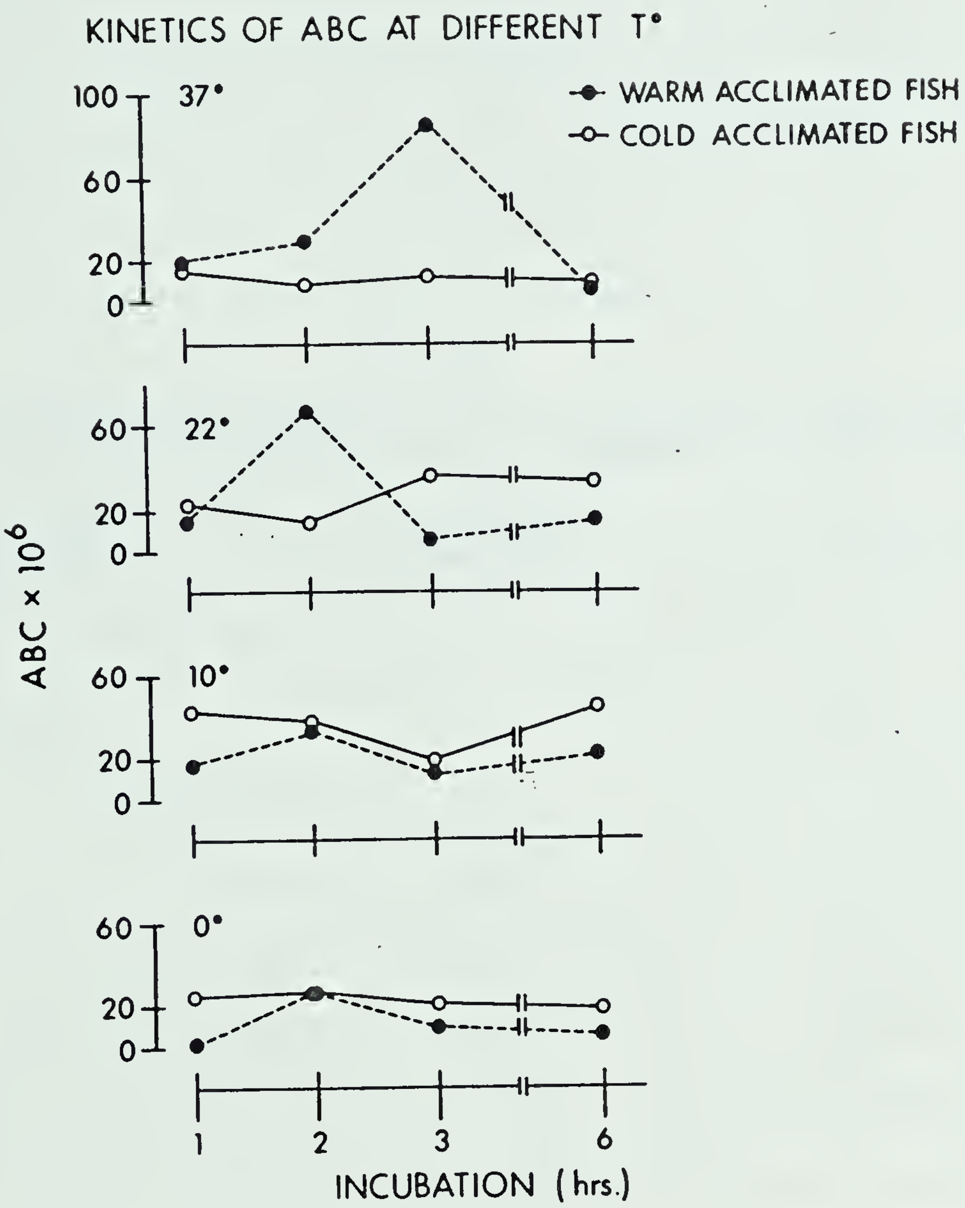

Fig. 2

Kinetics at different temperatures of the antigen-binding cells (ABC) of warm acclimated fish (--•--) compared with those of cold acclimated fish (—o—). The cells were incubated with 250 ng of POL for 2 hrs at 0^{o} and then for 1-6 hrs at the temperatures shown.

TABLE IV

RATE OF ANTIGEN BINDING AND CAP FORMATION IN MOUSE CELLS 6

Incubation	$ABC/10^{6}$ [b]	% Caps
0^{o} (1 hr)	0	-
" + 37^{o} (10 min)	24	55
" " (1 hr)	24	94
" " (2 hrs)	12	62
" " (4 hrs)	22	46
" " (6 hrs)	14	48

a) - the cells were incubated with 250 ng/ml of POL.

b) - 10^{6} refers to the number of screened cells.

c) - This table was adapted from Ref. (44) and (93).

dity of the suspensions. The majority of ABC were lymphocytes of different sizes in the range between 6 and 12 μ (Fig. 3, 4). Few macrophages were also labelled, but they were excluded from the reported data.

Antigen-induced capping and grain-count analysis. Redistribution of antigen-receptor complexes and capping were observed in different percentages of labelled cells, at all the temperatures and durations of incubation tested (Fig. 5, 6). At 10° and 22° almost all the labelled cells from cold-acclimated fish capped within 1 and 2 hrs respectively. At 0° 3 to 6 hrs were required for more than 77% of the labelled cells to cap, while at 37° the percentage of capping cells oscillated between 57.8% after 1 hr, 25% after 2 hrs and 60% after 6 hrs.

81% of the antigen-binding cells from warm-acclimated fish capped within 1 hr at 37°, but required 2 hrs at 0°. At 10° and 22° the ABC from these animals formed less caps, and later, than the cells from cold-acclimated fish. The chi-square test has shown that at each temperature of incubation the capping kinetics of the cells from differently acclimated fishes were independent (p less than 0.01).

A correlation was observed between all the percentages of capping ABC and the relative numbers of grains (Fig. 5), in which cap-formation appeared to be inversely proportional to the number of grains on the labelled cells (correlation coefficient: r = - 0.67, significance limits less than 0.001, confidence limits more than 99%). This correlation was observed in cells from both warm and cold-acclimated fishes but, while the correlation coefficient was very significant in relation to the cells from warm-acclimated fish (r = - 0.88), it was not so for those from cold-acclimated animals (r = - 0.29). The ABC from the latter ones had on the average a significantly lower number of grains than

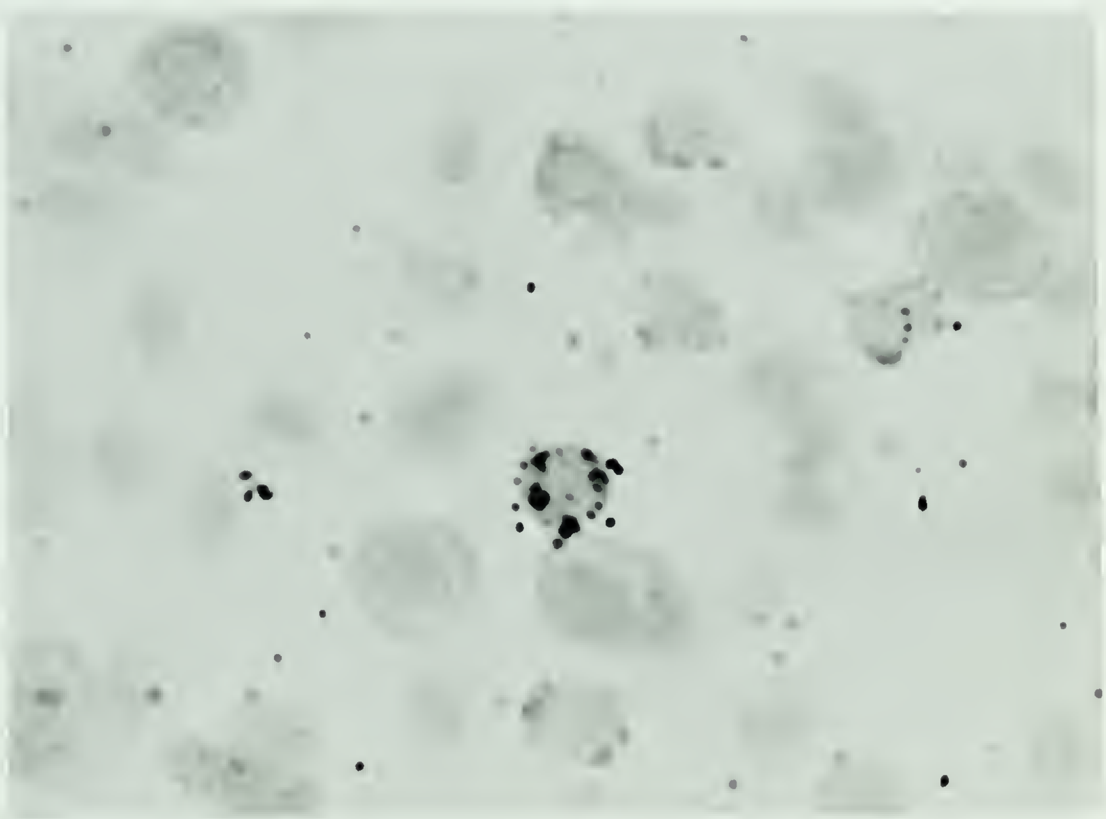

Fig. 3

Medium-size fish lymphocyte (9 μ in diameter) labelled with ^{3}H-POL. Gimsa stain. x 1200.

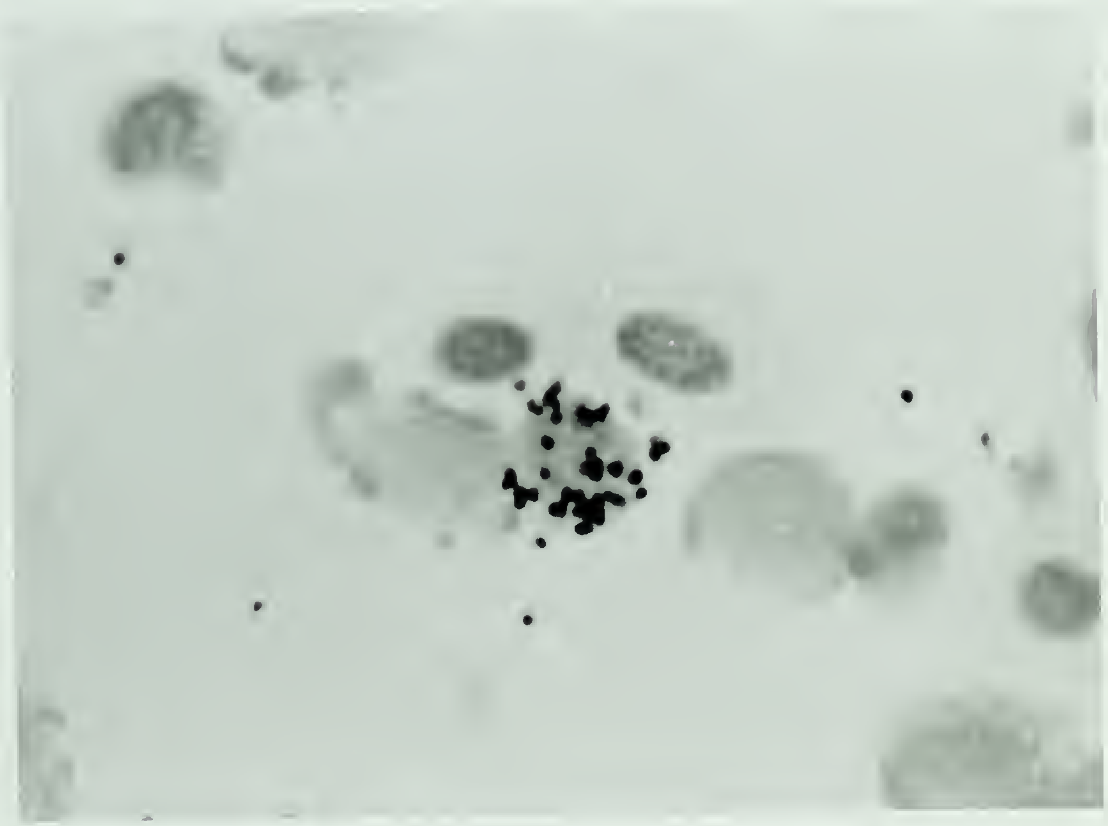

Fig. 4

Small fish lymphocyte (6 μ in diameter) labelled with ^{3}H-POL. Gimsa stain. x 2400.

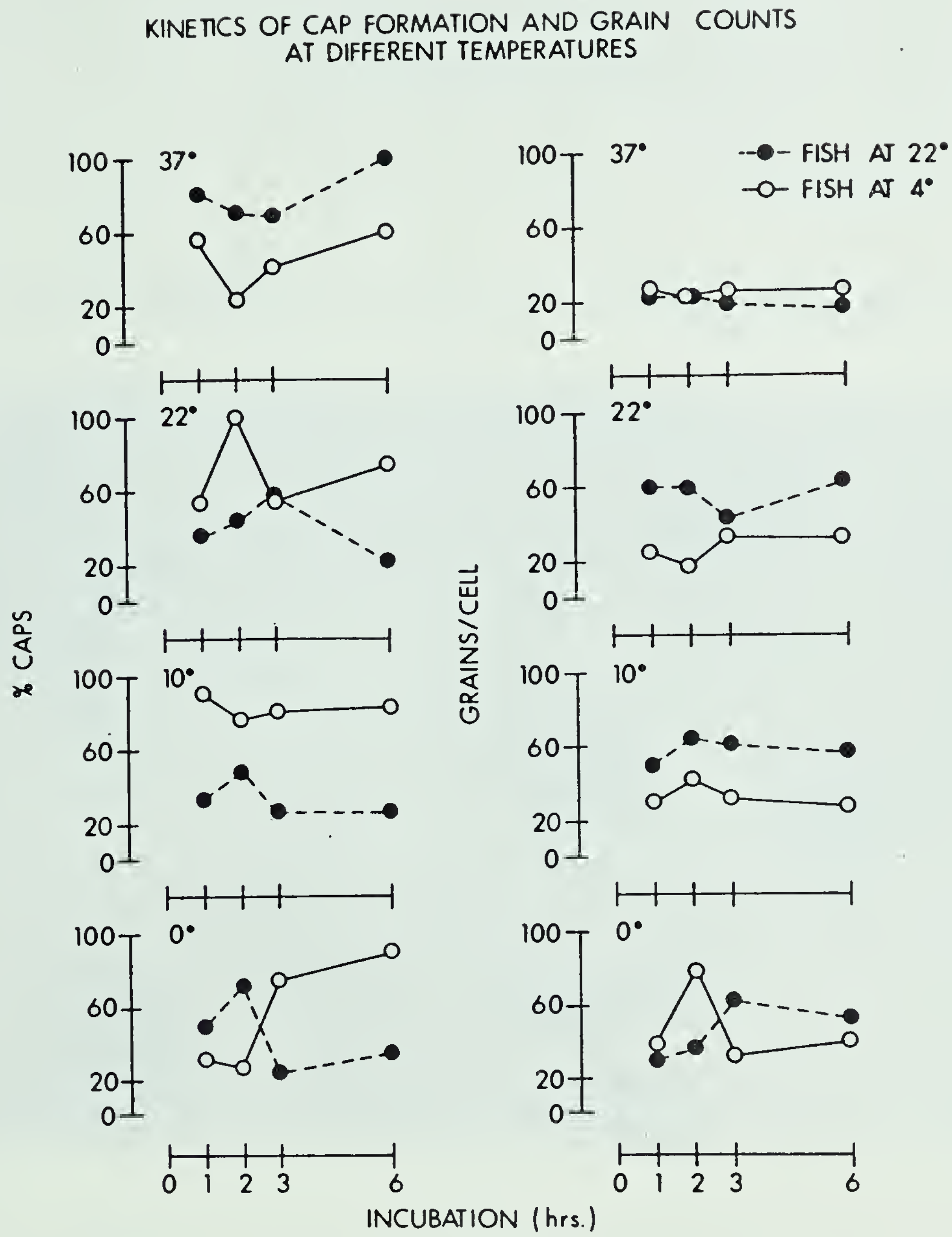

Fig. 5

Kinetics of cap formation and grain counts at different temperatures. The different symbols refer to the temperatures of acclimation of the fish. The dose of the antigen and the duration of the pre-incubation were as in Fig. 2.

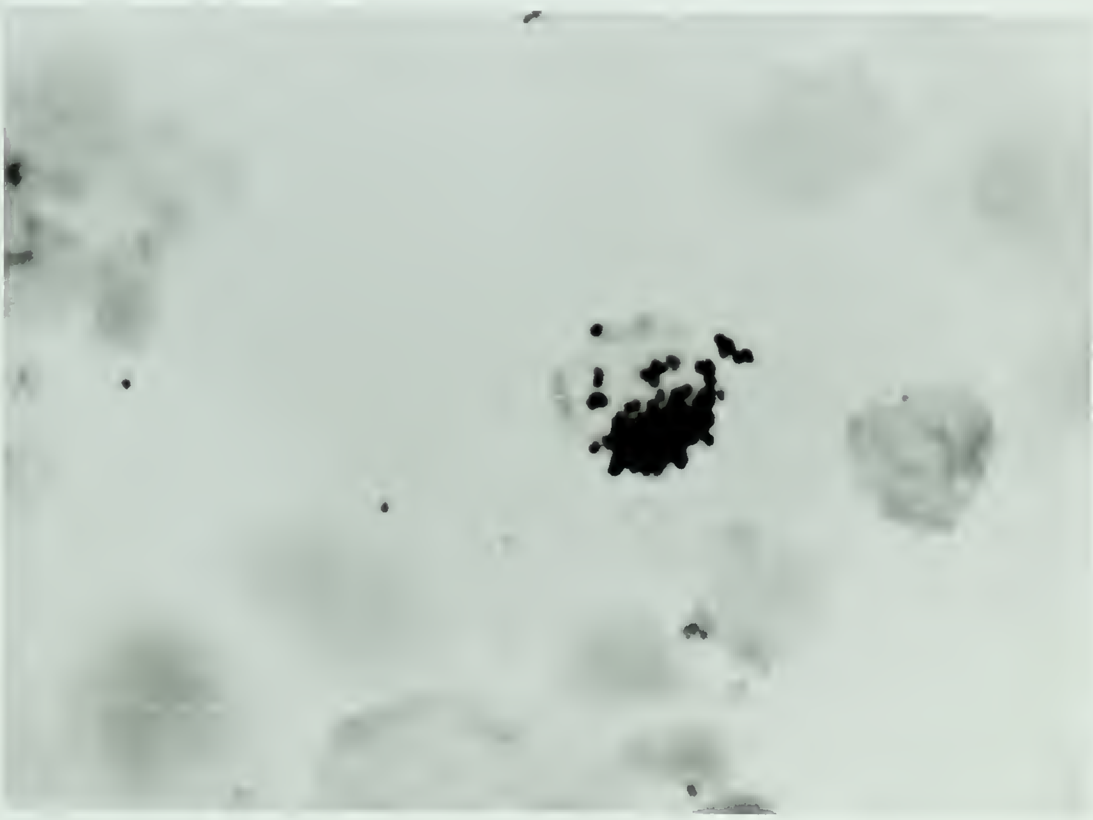

Fig. 6

Medium fish lymphocyte (9 μ in diameter) forming a cap with ^{3}H-POL. Gimsa stain. x 2400.

those from warm acclimated fish (p less than 0.05).

By contrast the experiments conducted on mouse cells have shown that they do not form caps at temperatures below 12° and large percentages of capping cells could be seen only at temperatures above 25° (Fig. 7, Table IV).

Fluctuations were observed in the number of ABC and in the percentage of caps after different periods of incubation from which it appears that, as in mice (44), the caps were shed from fish ABC and newly formed receptors became labelled with antigen.

Effect of acclimation on immunocompetence. Both warm and cold-acclimated fishes reacted to immunogenic doses of POL with parallel time-kinetics curves, while kept at the respective temperatures of acclimation (Fig. 8, 9). In warm-acclimated fish the peak number of AFC in the spleen was observed 3 weeks after immunization, while in fish kept at 4° it appeared one week later. There was no statistically significant difference in the numbers of AFC between the two groups of fishes. On the other hand, the peak titers of serum antibody were observed 3 weeks after immunization in both groups, but the response at 22° was significantly higher than at 4° ($p = 0.5$).

DISCUSSION

Goldfish lymphocytes can bind antigen and form caps within a wide range of temperatures between 0° and 37°. This is in contrast with the data gathered from experiments on mouse cells, which have shown that caps could be detected only after incubation at 37° (44).

The difference in antigen-receptor mobility at cold temperatures between fish and mouse cells can be explained in terms of different composition

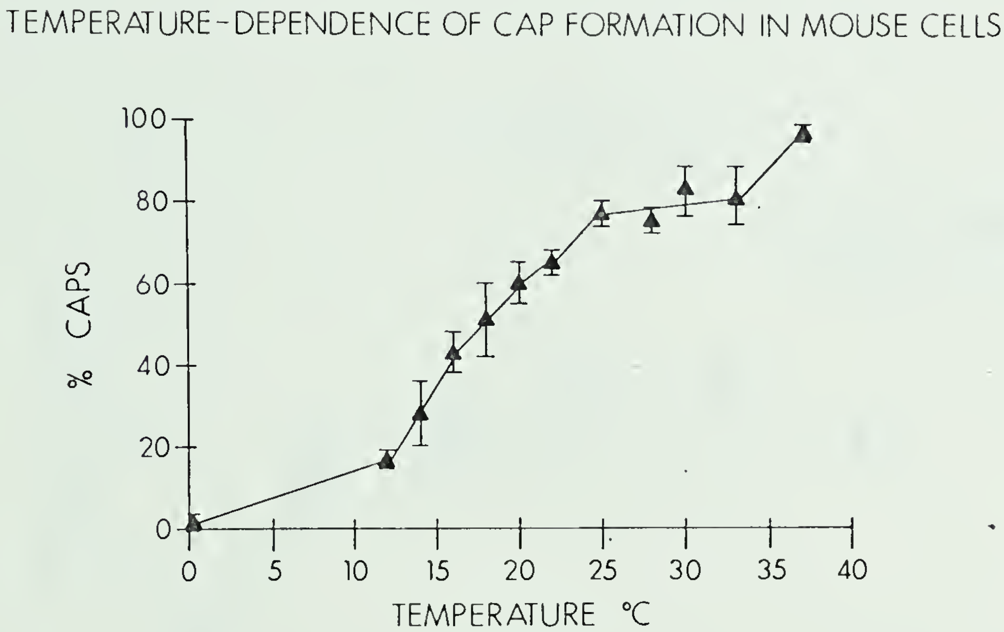

Fig. 7

Effect of temperature on cap formation in mouse spleen cells. These were coated with rabbit anti-mouse-IgM IgG and then incubated with fluorescent goat antibodies against the Fc of RGG. Each point shows the mean $\pm$ S.D. of the results from 3 or more mice.

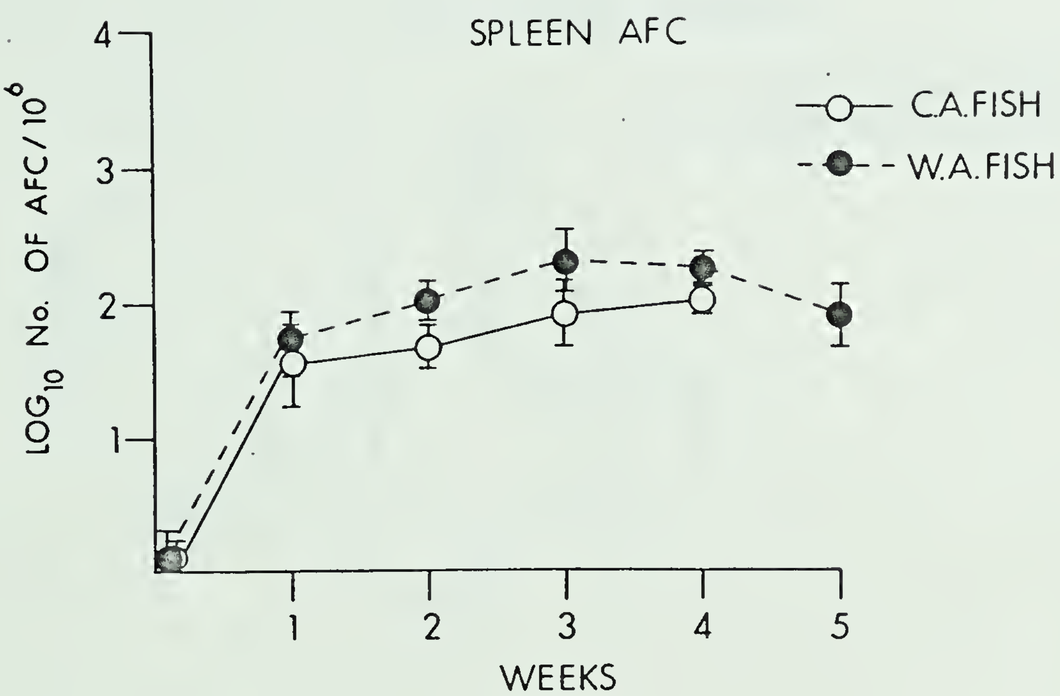

Fig. 8

Kinetics of the antibody-forming cells in the spleen of warm (--●--) and cold (—o—) acclimated fish. The animals were immunized with 10 ug of POL. Each point is the mean ± S.D. of 6 fish.

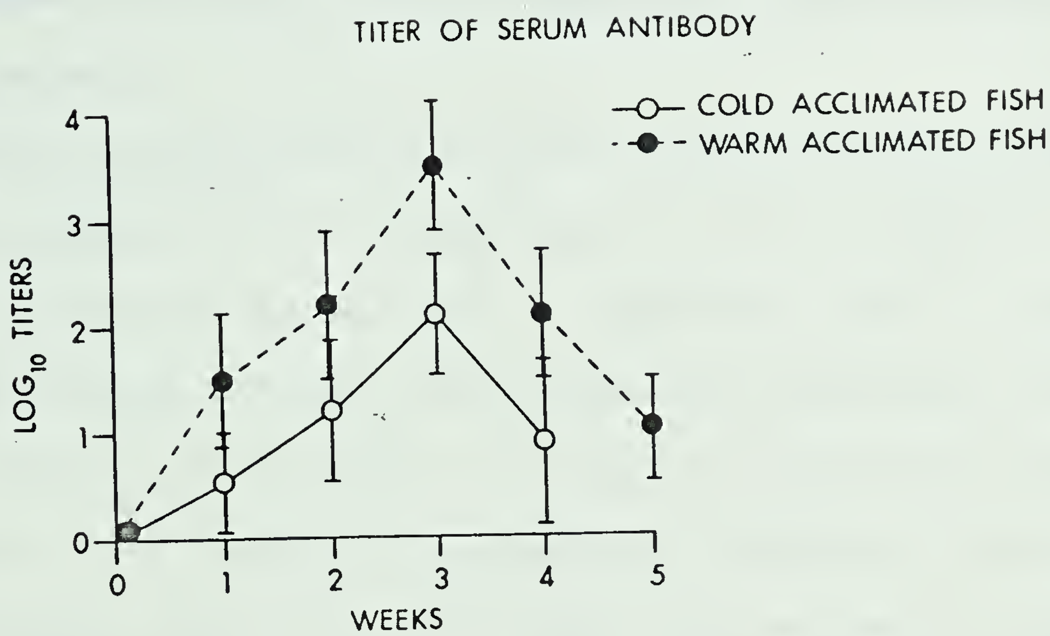

Fig. 9

Kinetics of the serum antibody titer in cold (—o—) and warm (--●--) acclimated fish after immunization with 10 ug of POL. Each point is the mean ± S.D. of the titers from 6 fish.

of the phospholipids in the membrane, which would result in different fluidity of the cell surface.

The temperature-dependence of cap-formation in mouse cells seems to reflect changes in the physical state of the membrane phospholipids, which have been attributed to phase-transitions from gel (below 12^{o}) to liquid crystallin (between 12^{o} and 30^{o}) to fluid (at 37^{o}) (90, 114). This observations are in agreement with the fluid mosaic model of the cell membrane (147) which is also supported by results obtained in other experiments.

Some mutants of E. coli cannot synthesize unsaturated fatty acids; when these are supplied in the growth medium, the rate of the Beta-glucoside transport system becomes dependent on temperatures that are characteristic of the type of fatty acid supplied (165). This experiment suggests therefore that the higher the content of unsaturated fatty acids, the higher is the fluidity of the membrane. The present observations are furthermore supported by the finding that the membrane lipids of Poikilotherms include more unsaturated fatty acids the lower is the temperature at which they are grown (66, 151).

The analysis of the kinetics shown in Table III and Fig. 5 suggests that in goldfish spleen cells capping is also regulated by the amount of antigen bound, as estimated from the number of grains overlaying the labelled cells (44). As shown by statistical analysis, a strong inverse correlation between percentages of capping and the relative numbers of grains was observed in ABC from warm-acclimated fish. The same correlation was not statistically significant in cells from cold-acclimated fish which, as a whole, appeared to bind less antigen than cells from warm-acclimated fish. The most likely explanation for these

differences seems to be that warm acclimated fish, which at 22^{o} have an almost optimally active metabolic rate (67), synthesize and exhibit more receptor sites. This in turn could have a negative effect on capping, since it has been shown that high density of receptors causes more antigen to be bound and tends to inhibit capping (44). In contrast, at 4^{o} the metabolic rate of this fish is very close to its standard one, and this seems to result in the formation, by the ABC, of a minimal number of receptors which can cap more easily after interaction with the antigen.

The fact that cold-acclimated fish can respond immunologically to antigenic stimulation at 4^{o} indicates that these Poikilotherms have retained the necessary biological apparatus that may have been lost by the Homeotherms in the process of their evolution.

CHAPTER III - KINETICS OF ANTIGEN-BINDING CELLS IN TOADS AND FISH DURING THE FIRST THREE DAYS OF IMMUNIZATION

MATERIALS AND METHODS

Animals. For these experiments goldfish *C. auratus* described in Chapt. II were kept at 22^{o} before utilization. Wild marine toads *Bufo marinus* of both sexes were obtained from commercial sources. The toads were kept at room temperature in plastic cages with the bottom covered with wet sawdust. No more than 4 toads were kept in each cage. They were fed one newborn mouse per week.

Immunization. For each experiment 5 goldfish were injected i.p. with 10 μg of POL from *S. adelaide* strain SW1338. One or more toads were immunized i.p. with 100 μg of the same POL.

Preparation of isotonic medium. Cells can be separated on the basis of their different velocity of sedimentation at unit gravity, which depends on their size (128). Therefore it is essential that in this procedure the cells maintain their natural volume, without swelling or shrinking, as may be caused by hypo- or hyperosmotic media. Such artifacts were avoided by adjusting the osmolarity of Phosphate-Buffered Saline (PBS) (50) and Fetal Calf Serum (FCS) (GIBCO) to that of fish or toad sera. The osmolarity of these and of PBS and FCS diluted with different concentrations of distilled water was measured at 22^{o} $\pm$ 2^{o} with a Hewlett-Packard model 302B vapor pressure osometer. Before use the instrument was left on for 24 hrs. NaCl solutions were used as standards for calibrations. Triplicate readings of unknown solutions were interspersed with recalibrations against a standard. Each result is the mean of at least 6 readings. Toad serum had an osmolarity of 210

mOsms, and accordingly PBS was diluted with 14% of H_2O; fish serum had an osmolarity of 240 mOsms and PBS was diluted with 11% of H_2O (Fig. 10).

Preparation of cells. The fish were sacrificed by cerebral concussion; the toads by pithing. The spleens were removed aseptically, minced with fine scissors and passed through a stainless steel sieve into isotonic PBS-3% FCS. The suspension were allowed to stand on ice for 10 min. to let large particles sediment. Then the cells were washed once in the same medium and counted in a hemocytometer. Cell viability was determined by eosin dye exclusion (75).

Cell separation. Spleen cells were separated by a method based on velocity-sedimentation differences at unit gravity (128). In this system the cells settle in a non-linear, shallow, density gradient composed of 5 to 30% FCS in PBS, known as "sta-put" (108). The gradient was generated by a system of three connected bottles (a,b,c,) containing the following volume of PBS and percentages of FCS, a) 400 ml-30%, b) 400 ml-15%, c) 50 ml-5%. Magnetic stirrers were used to continuously mix the contents of bottles b) and c), and the gradient was removed by gravity flow from bottle c) at a rate of 25 ml/hr. Since FCS can contain agglutinins for fish and toad cells, it was absorbed 3 x with 1.0 ml of packed toad or fish spleen cells for 30 min. at 37°, and then centrifuged at 1085 g for 10 min. A total of 10^7 white and red cells per ml were placed in the staput, in a volume of PBS-3%FCS not exceeding 45 ml. The glass staput used had a diameter of 16.5 cm. The cells were sedimented for periods of 2.5 hrs., at 4°, after which 15 ml-fractions were collected from the bottom of the staput. From each fraction 0.5 ml were removed for cell counts in a model B Coulter Counter fitted with

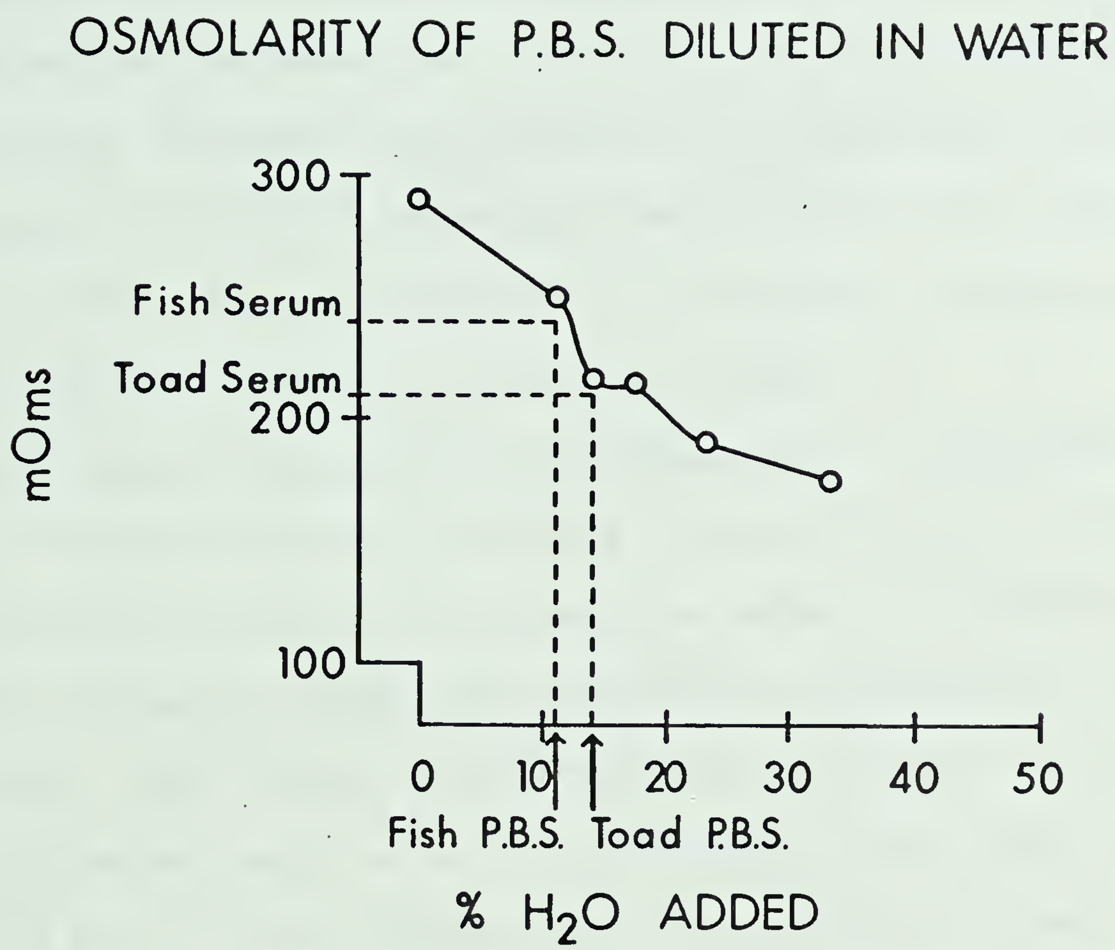

Fig. 10

Osmolarity of PBS diluted in water. Each point and the values for fish and toad sera correspond to at least 6 uniform readings.

a 70μ aperture. Differential counts of leuko- and erythrocytes and viability by eosin dye exclusion were determined in a hemocytometer, counting at least 100 cells per fraction. The fractions were centrifuged at 330 g for 10 min., the supernatants discarded, and the cell pellets resuspended in L-15 medium. Cells were pooled according to velocity of sedimentation. 20 x 10^6 cells of the original cell suspensions were kept unfractionated at 4^o in PBS-3%FCS. All cells were assayed for ABC, by incubating them at 0^o for 2 hrs. with 250 ng of ^{3}H-POL. They were then washed and processed for autoradiography, stained, and screened as described in chapt. II.

Calculation of the velocity of sedimentation. Calculations were made on a Wang 500 computer, utilizing a program previously elaborated by others(91) in which the sedimentation velocity is determined by dividing the distance for which each cell fraction sedimented by a time value. This time would include the time the cells in a particular fraction had spent in the cyclindrical part of the staput chamber, plus half the time they had spent in the conical section.

Cell recovery after velocity sedimentation. The total number of cells recovered in all fractions was compared with the number of cells in the unfractionated cell suspension, to assess the percentage of effectiveness of the separation procedure. The recovery of lymphocytes was 80-90% of both fish and toad cells, while that of the ABC was 60-100% in toad and 40-60% in fish cells.

Expression of the results. The results are expressed in terms of both percentages of that pool of fractions which gave the maximum number of ABC, and numbers of ABC/10^6 white cells. By the first method all responses were related to the peak reactivity of each experiment, facilitating

comparison between experiments (9). By the second method it was possible to observe quantitative kinetics in terms of actual number of ABC per 10^6 leukocytes present in the spleen of the tested animals. Each cell separation experiment was repeated at least twice.

RESULTS

Sedimentation velocity separation of toad cells. Before analysing the size kinetics after stimulation, it was important to have some background information about the population profile of spleen cells from normal toads. As shown in Fig. 11, the distribution of pooled spleen cells from 2 or more toads covered the almost complete range of sedimentation velocities, forming two major peaks corresponding to velocities of 2.4 and 3.6 mm/hr. Differential counts of the cells in each fraction have shown that the erythrocytes sedimented with the highest velocities and almost pure concentrations of leukocytes were obtained in a major peak corresponding to 3.6 mm/hr. Most of the lymphocytes sedimenting in these fractions were of the small and medium-size type; few smaller ones were observed in the 2-3 mm/hr range. The second peak obtained at these velocities contained mostly dead cells, the number of which appeared to be much higher that in the original cell suspension. Since the loss of cell viability in the stored unfractionated suspension was minimal (less than 5%), a cytotoxic interaction between cells from different individual toads, known to react in mixed lymphocytes cultures (MLC) (69), was suspected to be at least one of the causes of mortality. This hypothesis was tested by fractionation of cell suspensions from single spleens. Fig. 12 shows a typical result of one of these fractionations, which were repeated 3 times with constant results.

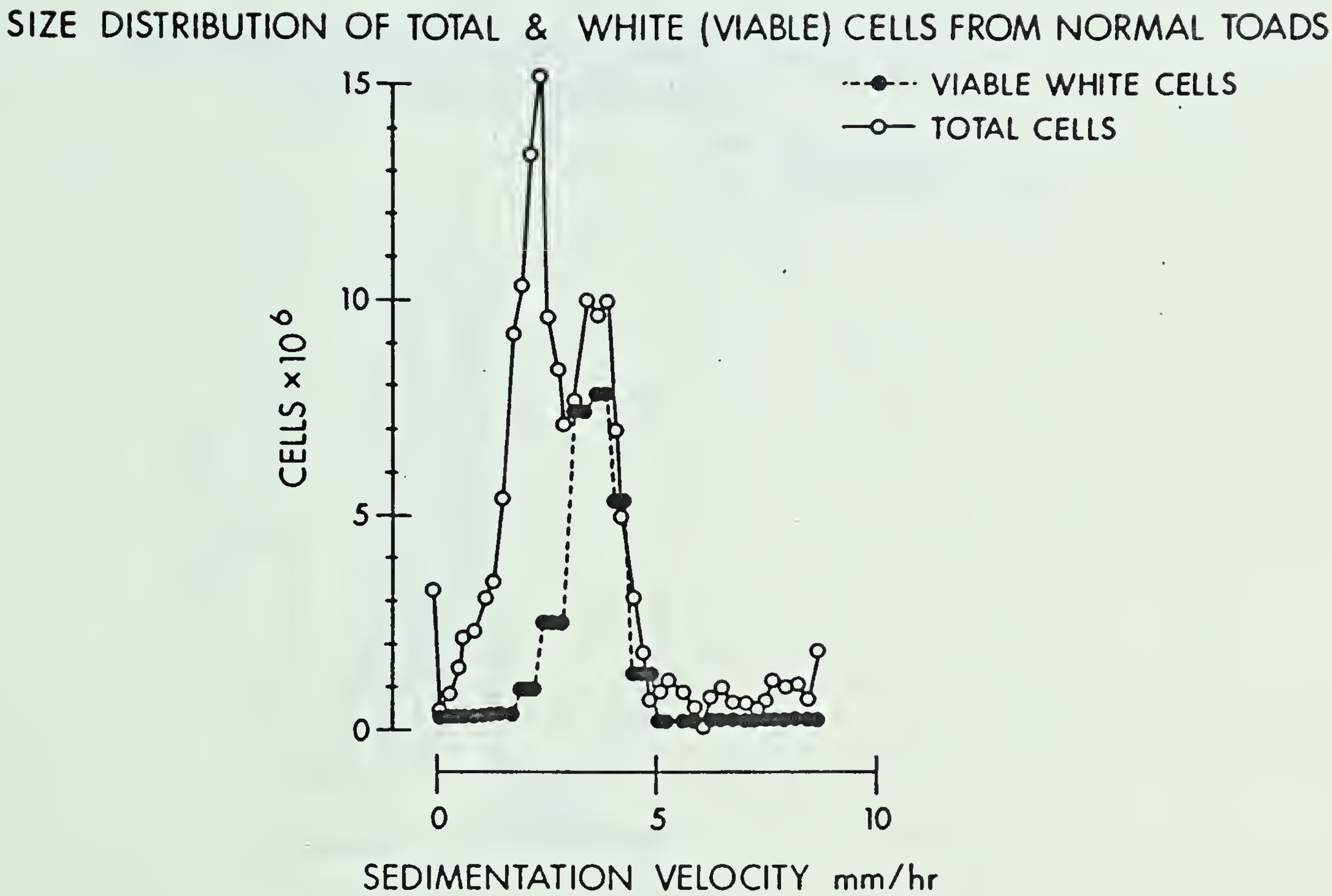

Fig. 11

Sedimentation velocity separation of total cells (—o—) and viable leukocytes (--•--) from a pool of two toad spleens. The sedimentation time was 2.5 hrs. This separation was repeated 3 times with little variation in the basic pattern, and a typical profile is presented.

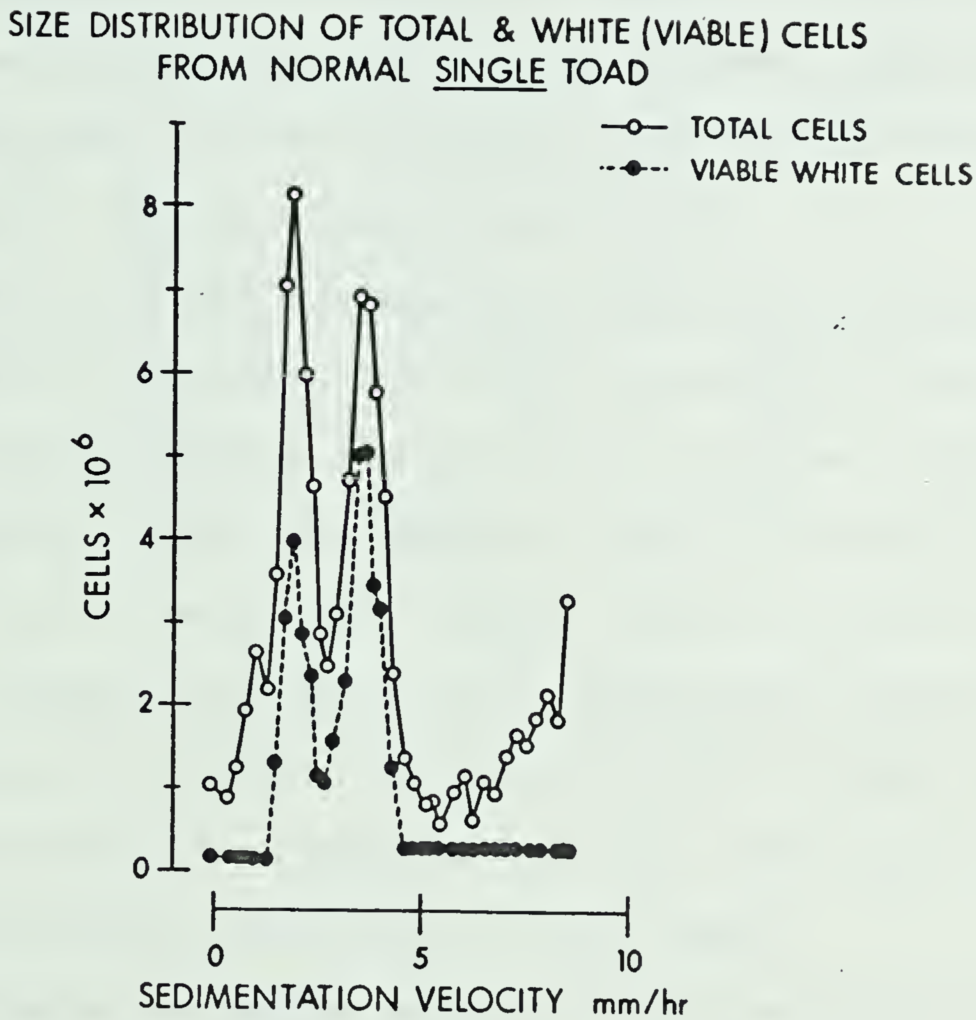

Fig. 12

Sedimentation velocity of the total cells (—o—) and the viable leukocytes (--•--) from single toad spleen. Note two peaks in typical profile of the viable leukocytes; it was obtained in 3 different separations. The sedimentation time was 2.5 hrs.

Two peaks of viable white cells were observed, one corresponding to a sedimentation velocity of 3.6 mm/hr, the other to 1.8 mm/hr. B. marinus spleens can vary considerably in size, independently of the body weight, and can contain from 30 x 10^6 to 230 x 10^6 viable leukocytes. Furthermore, no ABC were ever found in the cells sedimenting at less than 2.0 mm/hr. Therefore, for the following experiments in which more than 100 x 10^6 viable white cells were utilized, cells from two or more spleens were pooled before fractionation.

Size-kinetics of toad antigen-binding cells after immunization. After injection of the antigen, a presumably non-specific change was observed in the size-distribution profile of the total spleen leukocytes (Fig. 13). One day after immunization the peak relative to these cells had moved from 3.6 to 4.1 mm/hr and it had also widened to include more large cells. Two days after immunization a second peak of small cells was observed in correspondence with a velocity of 2.7 mm/hr, while the major peak had returned to sediment at 3.5 mm/hr. After 3 days the small cells were still present, even though in lower number.

Unfractionated toad spleen cells contained an average of 18 ABC/10^6, (range 13-24/10^6), before immunization. Their size-distribution followed that of the total population of white cells (Fig. 13, day 0). Most of the ABC had a diameter of 8-9 μ, while few were large cells with a diameter of more than 12 μ and very few were smaller than 8 μ. Cellular enlargement appeared one day after stimulation, when most of the antigen-binding activity was found in cells sedimenting in the 4-5 mm/hr range. One day later a new population of small ABC appeared, which sedimented at 2.7 mm/hr. At day 3 the small ABC had also undergone blastogenesis and only one broad peak of ABC was observed sedimenting at 4.9 mm/hr.

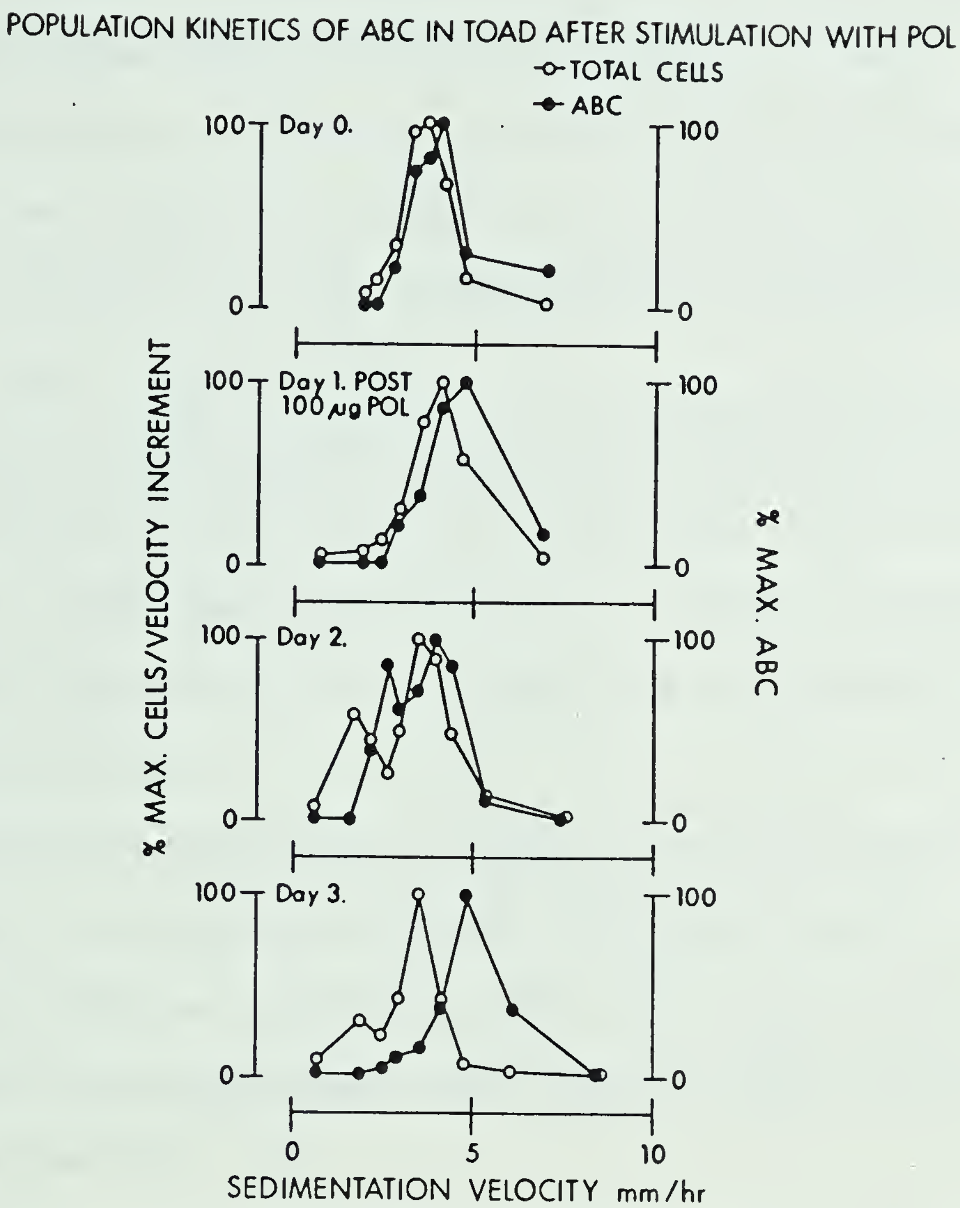

Fig. 13

Changes in toad spleen ABC velocity of sedimentation 0-3 days after immunization with 100 ug of POL. Each time-point was repeated twice using 2 or more spleens, except day 2, which was repeated 3 times, without variations in the profiles. The curves are normalized to the same peak height, regardless of the absolute ABC numbers in each fraction. Pools of 2 to 5 fractions sedimenting within 0.5-1.0 mm/hr were incubated with 250 ng of ^{3}H-POL for 2 hrs at 0^{o}.

The overall effect of immunization on the kinetics of the ABC was better seen by plotting the velocity of sedimentation vs. the actual number of ABC/10^6 screened cells (Fig. 14). The first observation derived from this graph is that the separation by velocity sedimentation was effective in concentrating most of the ABC in few fractions. Yet the most interesting indication is that ABC seemed to be less numerous one and two days after immunization, but reappeared in larger numbers and size after 3 days.

Morphology of toad antigen-binding cells. The appearance of toad ABC was not different from that of the same cells found in fish (Chapt. II) or in mice (44). Toad cells labelled with ^{3}H-POL could form caps at 4^o (Fig. 15), indicating that this could be a characteristic present in all Poikilotherms.

In preparations from immunized animals very few observations were made of couples of cells in which, even though they appeared to be in mitosis, only one of the two was labelled (Fig. 16). Three days after immunization most of the cells were either capping or were uniformly but heavely labelled (Fig. 17).

Sedimentation velocity separation of fish cells. A typical size-distribution profile of total spleen cells from normal fish is represented in Fig. 18. Fish erythrocytes sedimented in the fastest fractions, while the dead cells and the debris were found in the slowest ones. The majority of the leukocytes sedimented at a velocity of 2.25 mm/hr and were about 7 μ in diameter. Few larger cells sedimented at velocities in the 4-7 mm/hr range and were as large as 20 μ in diameter. Since numerous viable small leukocytes were present in correspondence of the slowest peak, no attempt was made to compare the sedimentation profile

NUMBERS & SIZE DISTRIBUTION OF ABC IN TOADS AFTER STIMULATION WITH POL

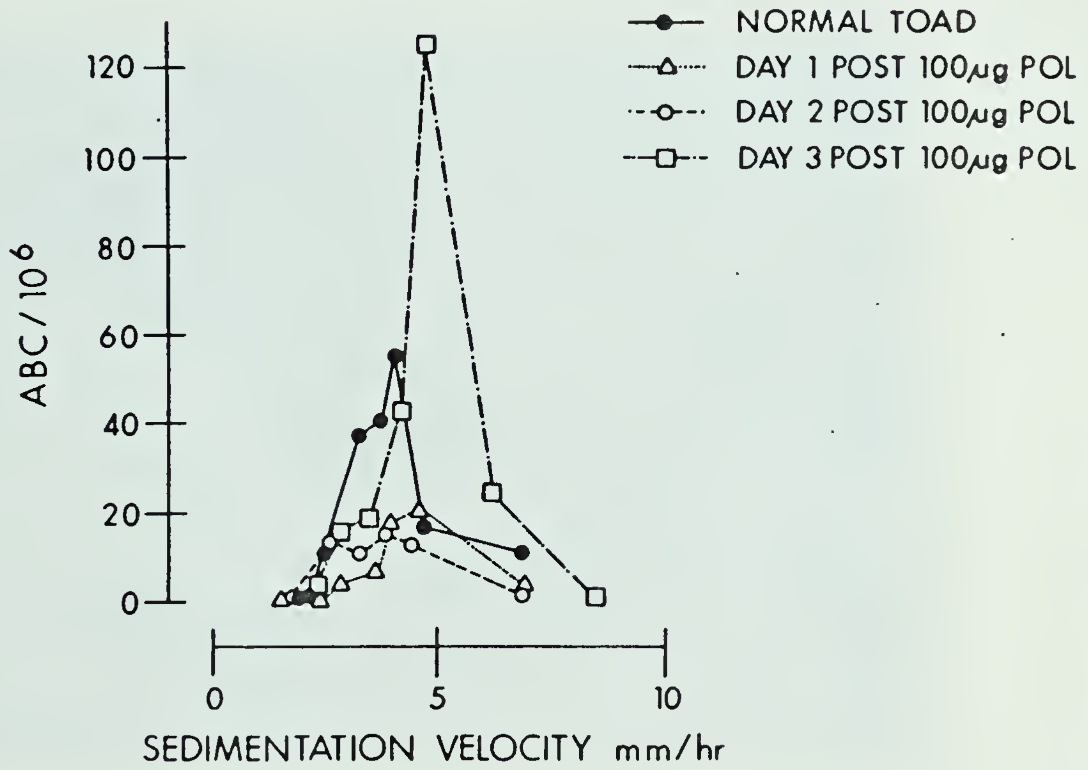

Fig. 14

Changes in number and velocity of sedimentation of toad spleen ABC 0-3 days after immunization with 100 ug of POL.

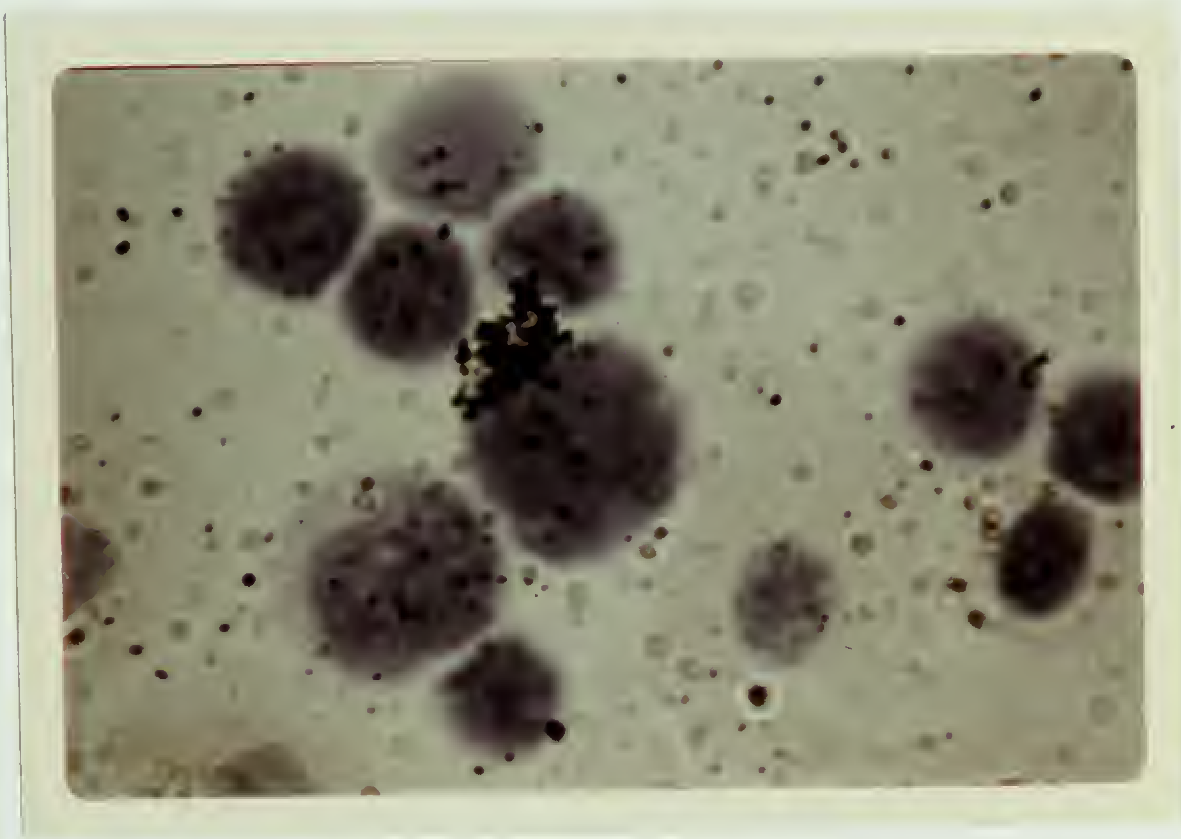

Fig. 15

Large toad lymphocyte (12 μ in diameter) forming a cap with ^{3}H-POL.
Gimsa stain. x 2400

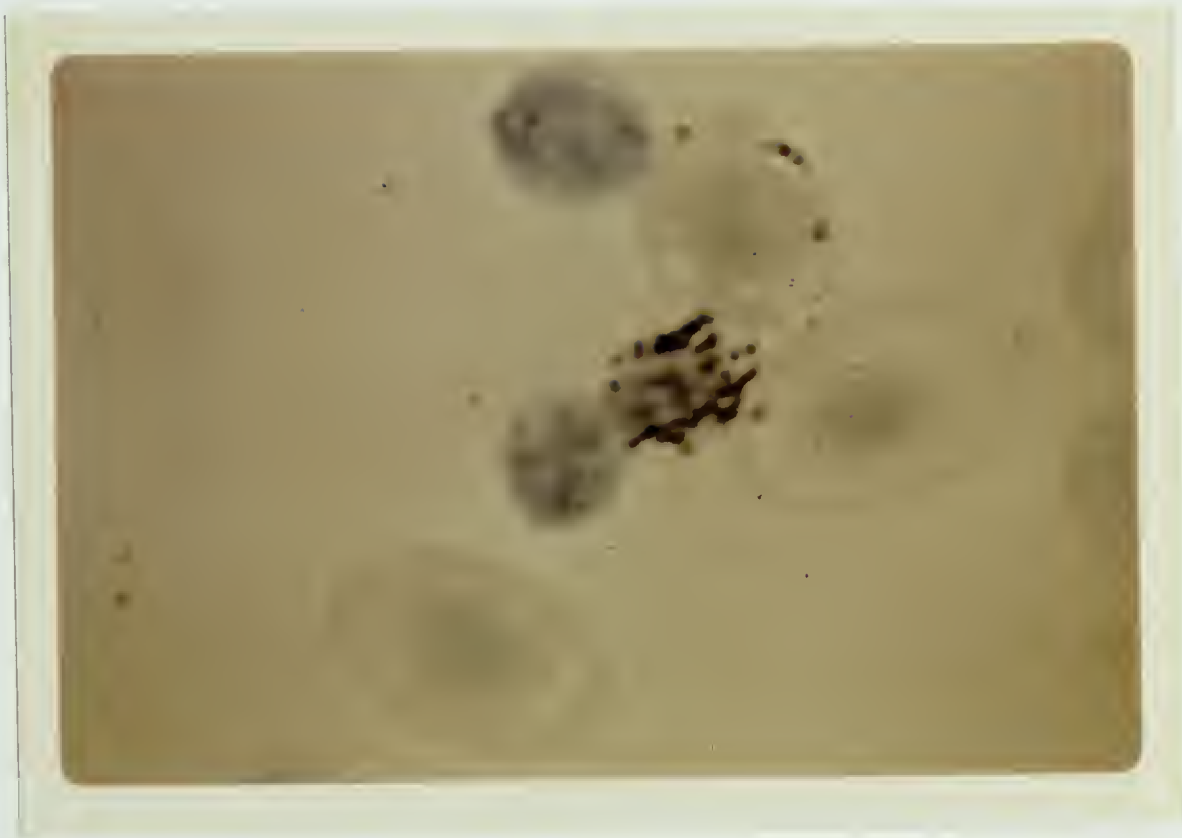

Fig. 16

Small toad lymphocyte (6 μ in diameter) labelled with ^{3}H-POL.
Gimsa stain. x 2400

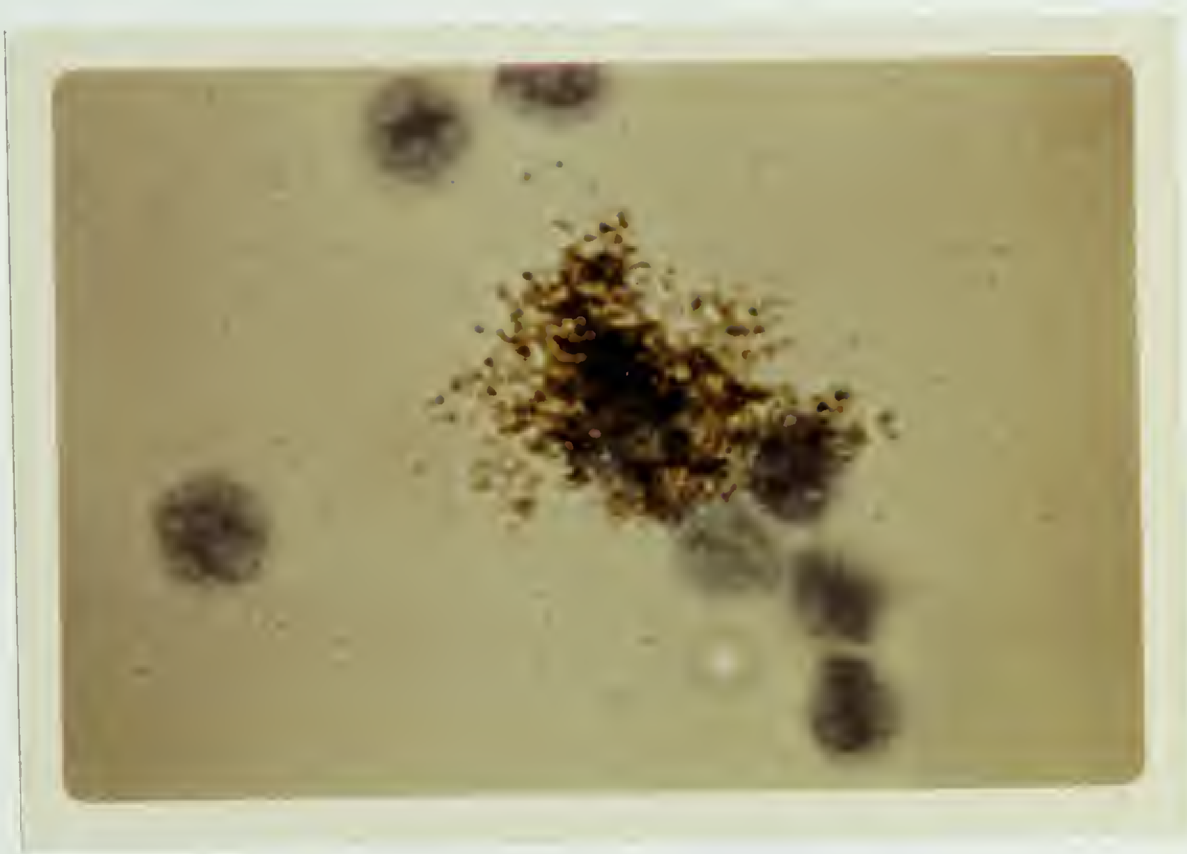

Fig. 17

Heavely labelled toad lymphocyte 3 days after immunization.
Gimsa stain. x 2400.

SIZE DISTRIBUTION OF TOTAL & WHITE (VIABLE) CELLS FROM NORMAL GOLDFISH

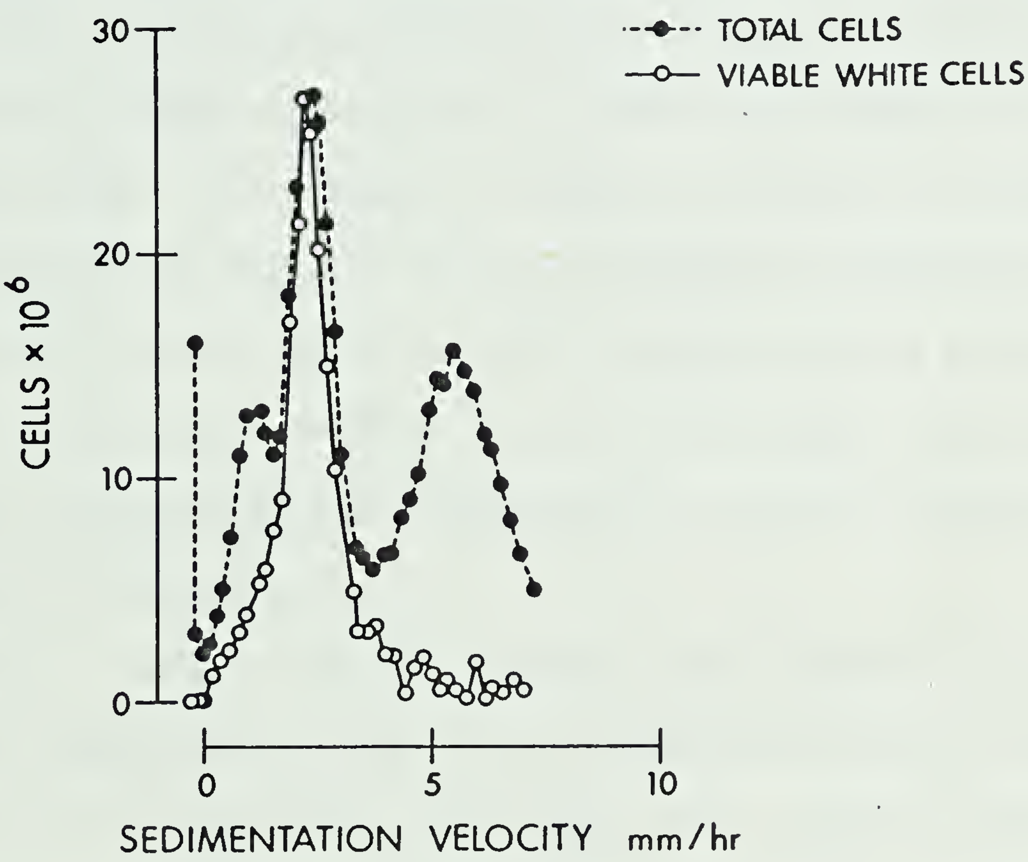

Fig. 18

Typical profiles obtained after sedimentation velocity separation of total cells (--•--) and viable leukocytes (—o—) from a pool of 6 fish spleens. The sedimentation time was 2.5 hrs.

of pooled cells with that of cells from a single fish.

Size-kinetics of fish antigen-binding cells after immunization. The non-specific effect of the antigen observed in toad spleen cells appeared to affect also fish cells, even though to a lower degree. In fact more cells were observed in the velocity range of 4-7 mm/hr 2 days after than before immunization. (Fig. 19).

Two sub-populations of ABC seemed to be present in the spleens of un-immunized fish, a major one with size and sedimentation characteristics of the rest of the splenocytes, and a smaller one composed of larger cells which formed a major peak at 4.0 mm/hr. One day after immunization most of the small ABC appeared to have enlarged and sedimented as a major peak at 4.0 mm/hr. After two days very few ABC were still small, while most of them had velocities in the 4.4-7.8 mm/hr range. After 3 days most of the ABC were of the medium size and more smaller ones had reappeared (Fig. 19).

The increase in number of ABC/10^6 screened cells observed in toad spleens 3 days after immunization seemed to have different kinetics in fish spleens. As can be seen in Fig. 20, 2 days after antigenic challenge there was a two-fold increase in the number of ABC/10^6, which neverthe-less one day later returned to values similar to those observed in spleens from normal fish.

DISCUSSION

Velocity sedimentation at unit gravity is a separation technique that mostly reflects cell size. Originally it was used to separate mammalian cells which had different functional properties (109, 128, 129).

The use of this procedure in the present study was initiated for two

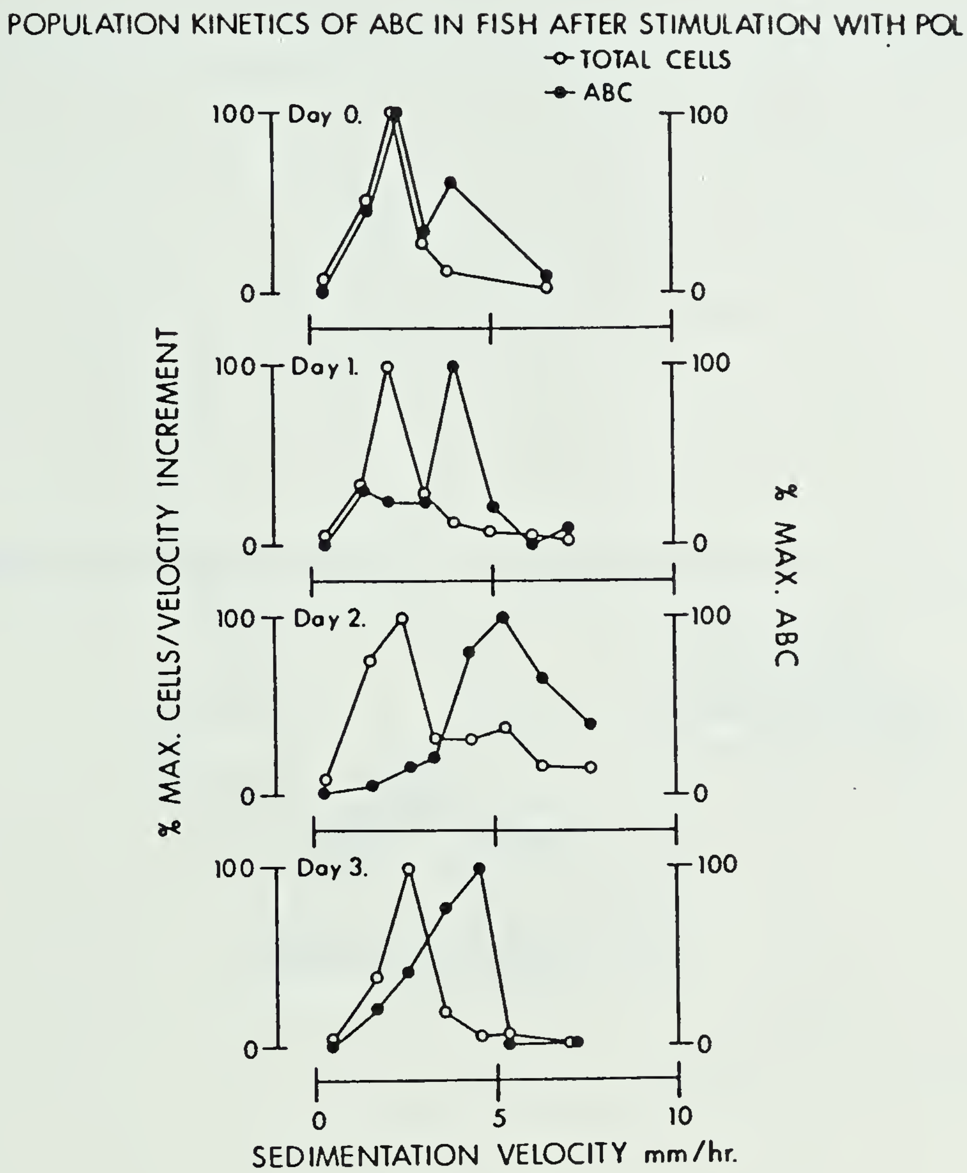

Fig. 19

Changes in fish spleen ABC velocity of sedimentation 0-3 days after immunization with 10 ug of POL. Each time point was repeated twice using the spleens from 6 fish, with little variation in the basic pattern. The assay for ABC and the presentation of the data are the same as in Fig. 10.

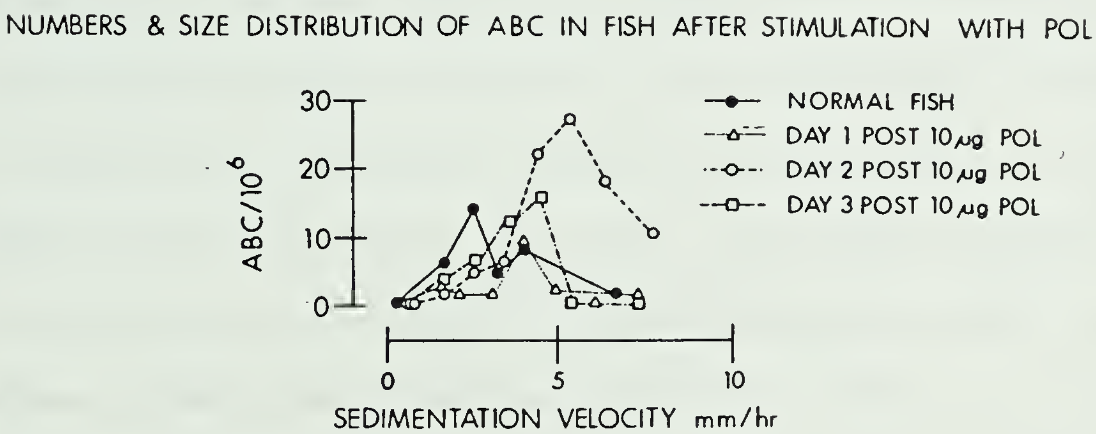

Fig. 20

Variations in numbers and velocity of sedimentation of fish spleen ABC 0-3 days after immunization with 10 ug of POL.

reasons. The first one was to establish an experimental continuity in the analysis of cell differentiation in toad spleen between the moment of immunization and the development of the AFC, which had been investigated previously (91). Secondly, the finding that in mice both ABC and immunocompetent cells coincided in blastogenic activity 24 hrs after antigenic stimulation (93) had made it phylogenetically interesting to compare this response with that of more primitive species such as B. Marinus and C. auratus.

The data reported here connect well with those obtained in another study on the differentiation of toad AFC which, 3 days after antigenic stimulation, had been shown to be mainly of the medium and large size types, had low density and most of them incorporated ^{3}H-Thymidine (91). At that time of the immune response the size profile of ABC had clearly moved towards the zone of the larger cells which coincided with that of the earliest detectable AFC. Furthermore, the number of ABC in the spleen of immunized toads was found to be double or more than before immunization, suggesting that cell proliferation had been active. Therefore it appears that, 3 days after stimulation, the two populations of ABC and AFC tend to coincide.

The results observed at day 1 and 2, following the antigenic stimulus, were rather unexpected. Even though the size distributions indicated that cell differentiation had occurred, as shown by the new peak of small ABC present at day 2, the proportion of large cells was higher at day 3 than at day 1. Furthermore, the actual number of ABC was lower at days 1 and 2 than before immunization. A possible explanation for these apparently contradicting observations could be derived from the results obtained by other authors (91). They found that in toads

the maximal AFC-response was reached faster in the blood than in the spleen, and early after immunization the AFC found in the blood were of higher density than those present in the spleen, suggesting that in toads the function of antibody-production matured in the blood earlier than in the spleen. In addition, those authors had some evidence that the differentiation of the AFC in these animals involved a series of halving divisions, without intermediate growth. On this basis it appears that, while the peak of small ABC at day 2 could have derived from divisions of ABC found at earlier times, the numerical results of such an event would be difficult to detect because of migration of immunocompetent cells from the spleen to the blood.

The same pattern of differentiation of ABC appeared also to be present in the spleen of goldfish, with some minor differences from that found in toads.

Two sub-populations of ABC were already present in the spleen of normal fish. The first effect of immunization appeared to be the loss of large ABC from the spleen and the enlargement of the small ones. Blastogenesis seemed to occur 2 days after immunization, in contrast with the data obtained from the toad, where it occurred at day 3. The earlier response of the fish, when compared with that of the toad, may be due to the fact that at room temperature the metabolism of the fish is more active than that of the toads, which at 37^{o} are known to produce a better AFC-response than at 22^{o} (43).

An observation that demands confirmation through specific investigations is that of cell couplets in which only one cell became labelled with tritiated antigen, even though their morphology was very similar to that found in the telophase of mitosis.It is normally accepted that,

after stimulation, the immunocompetent cells divide equally. Nevertheless the existence of unequal cell divisions has to be kept in mind, in view of phenomena such as memory cell production.

CHAPTER IV - KINETICS OF SERUM ANTIBODIES IN THE PRIMARY AND SECONDARY RESPONSES OF THE TOAD *Bufo marinus*

MATERIALS AND METHODS

Animals. Adult *B. marinus* toads were acquired and maintained as mentioned in Chapt. III. The animals were kept at room temperature, except for one experiment at 37^{o}, referred to in the text.

Groups of toads were matched in body weight, which on the average was 250 $\pm$ 50 gr. They were fed 1-2 baby mice per week. Care was taken to isolate those toads which would not eat in competition with others; they were fed separately or replaced. During the experiment conducted at 37^{o} the animals were fed twice/week.

Antigens. Polymerized flagellin (POL) from *S. adelaide* was prepared as described in Chapt. II. Sheep erythrocytes (SRC) were collected in Alsever's solution and stored at 4^{o}. Before being injected they were washed 3x and resuspended in the same solution.

Immunizations. Four groups of 4 toads each were injected with 1.0 ng, 100 ng, 10 µg and 100 µg/toad of POL in 0.2 ml of water. After 66 days the first 3 groups were reinjected with the same dose of antigen that was given for the priming immunization.

Two groups of 4 toads each were injected with 2 x 10^{8} SRC in 0.2 ml. 144 days thereafter one group received a second dose of 10^{8} SRC in 0.1 ml and the second goup received 5 x 10^{8} SRC in 0.5 ml. 44 wks after the first injection (23 wks after the second one) all toads of both groups were injected with 10^{8} SRC. All injections were made i.p..

Each experiment was repeated at least twice.

Bleeding. Normal and immunized toads were bled at weekly intervals by heart puncture using individual tuberculin siringes with 25 gauge needles.

Initially it was attempted to anesthetize the toads with Ms-222 tricane methane-sulphonate (Sandoz Biochem., Hanover, N.J.), with ether or by exposure at 4°. All these procedures were time-consuming and resulted either in high mortality or in inflation and rigidity of the ventral body-wall which made the cardiac puncture impossible. Since it was essential to keep the toads in good health for long-term experiments, cardiac punctures were attempted without anesthesia. To this procedure the toads reacted with little to moderate agitation for a few seconds when put in dorsal decubitus, but otherwise they underwent the puncture without excessive disturbance.

A volume of 0.1-0.15 ml of blood was taken from each animal and individually stored in Beckman microcentrifuge plastic tubes. The blood was incubated at room temperature for 30 min, followed by exposure to 4° for 1 hr and the sera were separated by centrifugation.

The sera from toads immunized with POL and those from control unimmunized animals were decomplemented by further incubation at 56° for 30 min. Sera from animals injected with SRC were only occasionally decomplemented as indicated in the text. All sera were tested for antibody activity on the same day of collection.

For serum fractionation experiments the toads were killed by pithing and bled from the heart with a pasteur pipette. The blood was collected in plastic Falcon tubes, incubated for 30 min at room temperature and at 4° for 1 hr, and the sera were separated by centrifugation in the cold.

Tests for humoral antibodies. The sera from POL-immunized toads were titrated for bacteria-immobilizing antibodies as described in Capt. II. Sera were titrated for SRC-hemagglutinins in titration trays with U-shaped wells, using capillary diluters (Biocult Linbro, Paisley, Scotland).

5×10^7 SRC in 50 µl were added to 50 µl of diluted sera and the trays were incubated at room temperature for 2.5 hrs before reading.

All sera treated with 2-ME (0.1 M) were incubated for 1 hr at 37^o before titration.

Fractionation of sera. Normal and anti-POL immunoglobulins were fractionated from pooled sera of toads either normal or immunized i.p. 14 days before with 100 ug of POL.

The immunoglobulins were first separated from the sera by precipitation with 50% sat. $(NH_4)_2SO_4$ at room temperature (76). The precipitate was collected by sedimentation for 15 min at 12100 g. The pellet was washed once in 50% sat. $(NH_4)_2SO_4$, dissolved in PBS and fractionated by gel filtration in PBS. This was done at 4^o on Sephadex G200 (Pharmacia, Uppsala, Sweden) in a column 5 cm in diameter and 95 cm high. Samples of 8-10 ml were loaded and 15 ml fractions were collected from the top, using a peristaltic pump at a flow rate of 55 ml/hr. The optical density was measured at 280 mu. Fig. 21 shows a typical result of gel filtration on Sephadex G200 of the serum proteins previously precipitated in 50% sat. $(NH_4)_2SO_4$. The fractions 40 to 57 obtained from the first separation were regularly gel filtrated once more to separate from IgM the macroglobulin aggregates and the contaminant IgG which appear as shoulders of the single peak in fractions 40-44 and 58-78 (Fig. 22).

The proteins were concentrated from pooled fractions by precipitation in 50% sat. $(NH_4)_2SO_4$. After centrifugation the precipitate was dissolved in 5 ml of 0.9% NaCl and dialysed for 24 hrs against 2 l of saline to remove the remaining ammonium salt.

The protein concentration was determined by absorbancy at 280 mµ, taking $E^{1\%}_{0.5cm} = 7.0$ for comparative purposes. Before being injected, both nor-

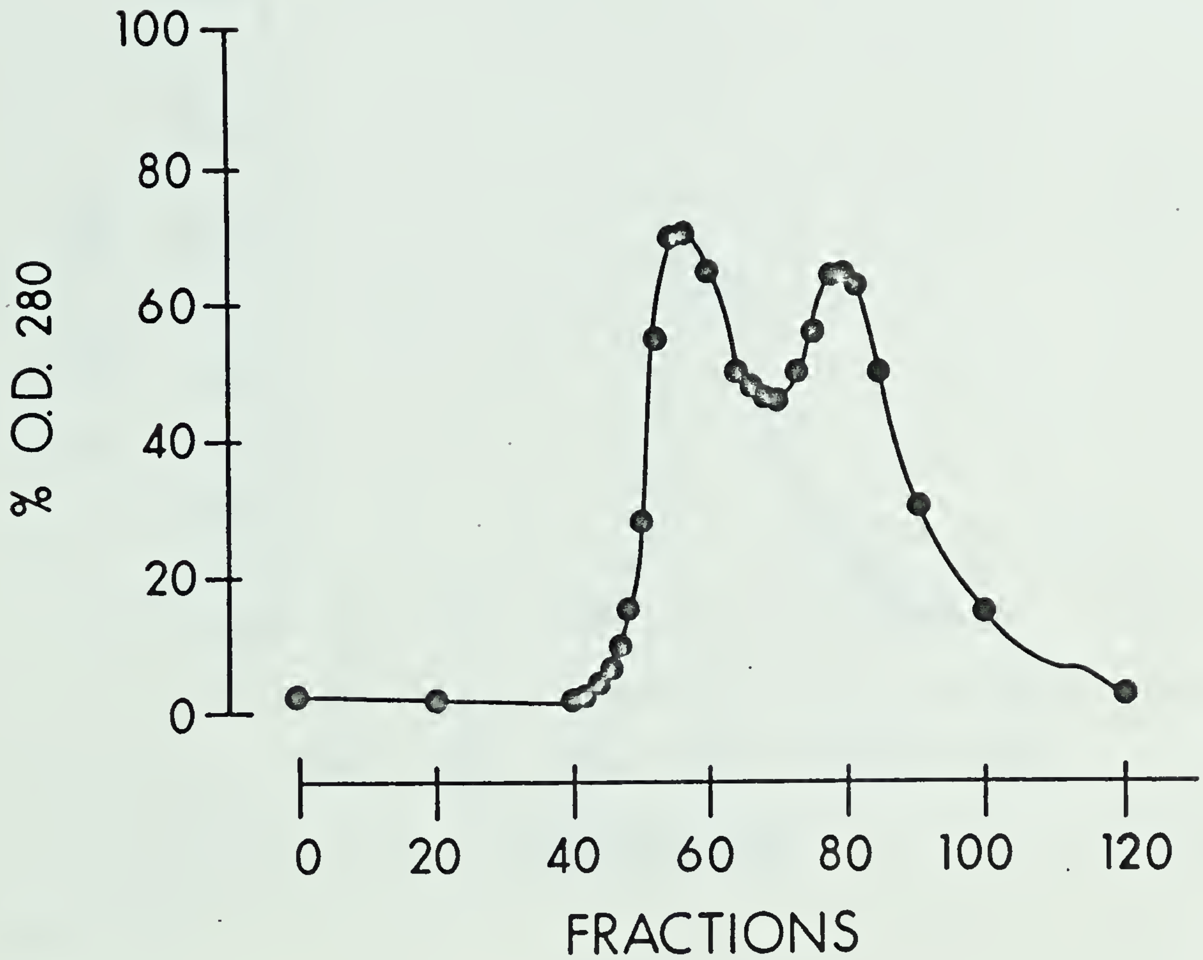

Fig. 21

Gel fractionation on Sephadex G-200 of the fraction of B. marinus serum precipitated with 50% sat. $(NH_4)_2SO_4$. This basic pattern was obtained several times with precipitates from both bormal and anti-POL sera.

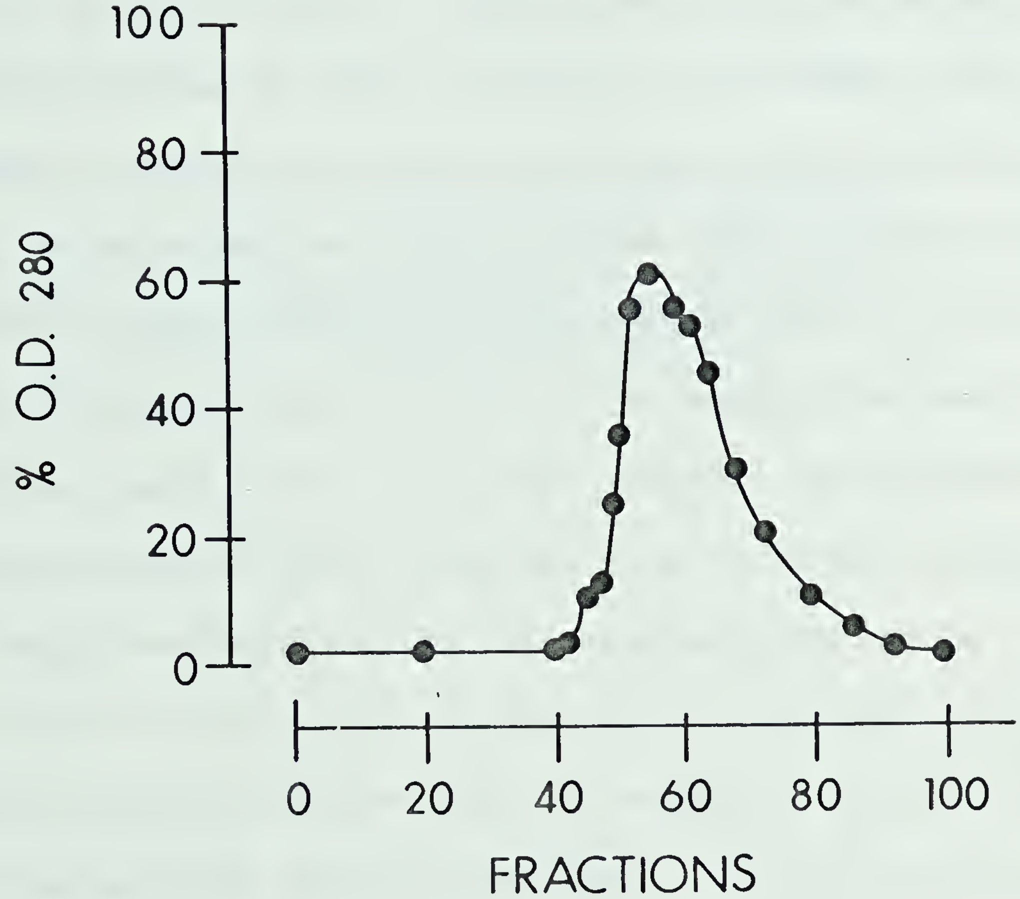

Fig. 22

Refractionation on Sephadex G-200 of the proteins in the pooled fractions 40-57 of Fig. 21.

mal and immune IgM and IgG were tested for bacteria-immobilization activity, as mentioned further in the text.

Immunoelectrophoresis. The purity of IgM concentrated from fractions 45-57 (Fig. 22) was tested by immunoelectrophoresis. This procedure was carried out in a cassette electrophoresis cell (Analytical Chemists Inc., Palo Alto, Calif.), using Barbital buffer at pH 8.6 (0.05 M with 0.035% ethylene-di-amino-tetraacetic acid (EDTA)). The samples were loaded on ready-to-use Agarose Universal Electrophoresis films (ACI) and the apparatus was run for 45 min. Then the proteins were precipitated by immuno-diffusion at room temperature for 12 hrs using a rabbit anti-toad-whole-serum antiserum. The precipitates were stained with 0.2% w/v Amido Black in 5% acetic acid for 15 min, washed with the same concentration of acetic acid and dried at 75-85^{o}. As shown in Fig. 23, a single precipitation line was obtained with IgM (no. 4 and 5) and with IgG (no. 6), while the preparation obtained after salt-precipitation still included both slow and fast moving proteins (no. 3). No. 1 and 2 are the patterns obtained with whole toad sera and are included for comparison.

Preparation of rabbit anti-toad-whole-serum antibody. Adult rabbits were given several injections of 3 ml of toad serum in CFA. The injections were made in small doses at several places each along the abdominal wall and in the interscapular region. The animals were bled 7 days after the last injection and the blood collected in glass tubes. After incubation for 1 hr at room temperature and for 2 hrs at 4^{o} the serum was separated from the blood by centrifugation.

Iodination of Toad-IgM. Aliquots of 3.5 mg of IgM from normal toads (N-IgM) were labelled with ^{125}I (New England Nuclear, Boston, Mass.) by

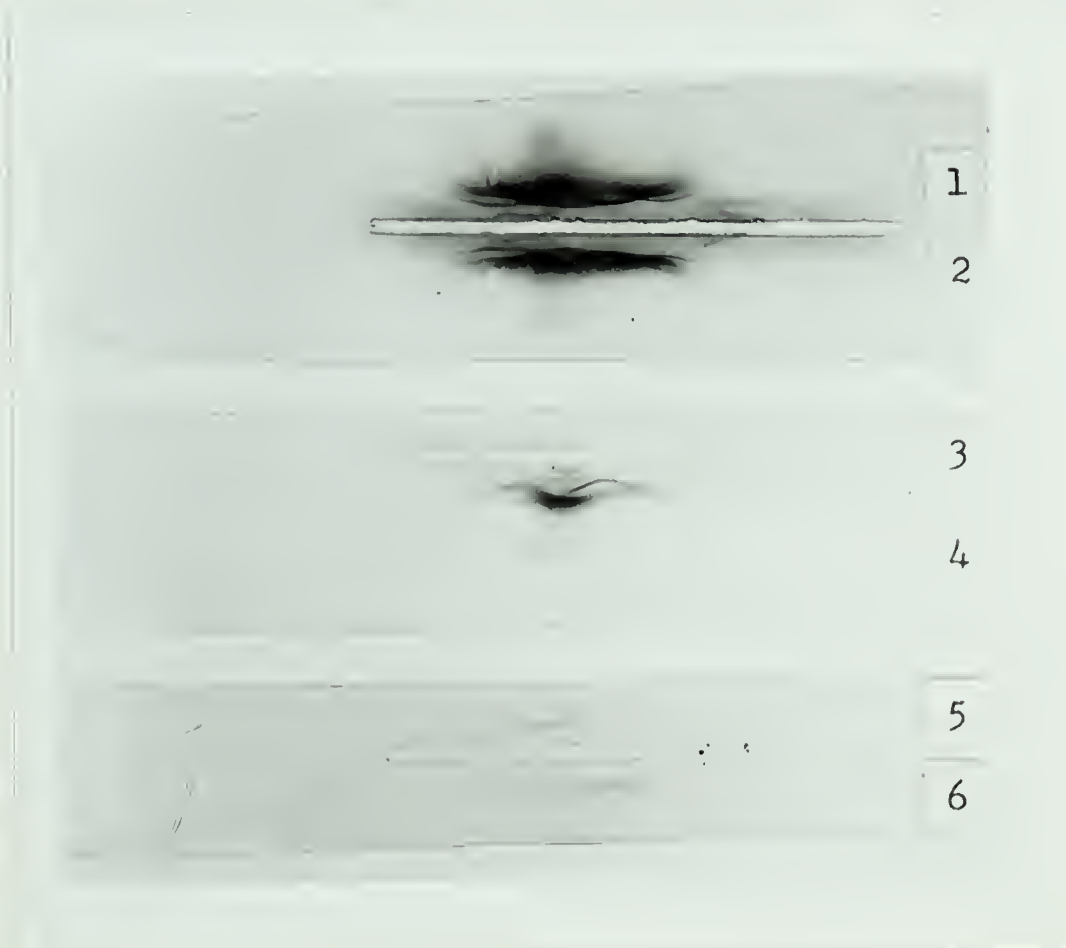

Fig. 23

Immunoelectrophoresis of toad whole serum (1,2), IgM (4,5), IgG (6) and the serum fraction precipitated with 50% sat. $(NH_4)_2SO_4$. All the immunodiffusion precipitation lines were obtained with rabbit antiserum against whole toad serum. The electrophoresis was run for 45 min, the immunodiffusion for 12 hrs. The catode is to the left.

the chloramine-T method (82). The effectiveness of labelling was measured by counting the trichloroacetic acid (TCA)-precipitate and the supernatant of a 0.1 ml sample immediately after labelling, 30 min later and again after dialysis for 12 hrs against 2 lts of saline. The TCA-precipitation showed that more than 99% of the label was in the precipitate. After 12 hrs of dialysis 88% of the radio-activity was associated with the proteins in the sample. All counts were made in a Packard Auto-Gamma Spectrometer.

Determination of the half-life of N-IgM in toad sera. Two groups of 4 toads each were kept at 22^{o} or 37^{o} for one week before the injection i.c. of 0.75 mg of ^{125}I-N-IgM in 0.3 ml. The toads were bled by cardiac puncture before the injection and thereafter, at 30 sec, 3, 6, 9, 24 hrs, daily for 6-8 days and, following this, every second day. 0.2 ml of blood was collected from each toad and the sera separated as described above. For the earliest counts 50 µl of serum, its TCA-precipitate and supernatant and 0.1 ml of whole blood from each toad were separately counted for radioactivity. After 6 hrs 50 µl of serum and 0.1 ml of whole blood were counted. All counts were adjusted for decay using a table in which the half-life of the isotope used is shown to be 60 days (48).

The mean radioactivity of the sera was plotted against time and the half-life of the injected protein was determined graphically from the slope of the semi-logarithmic curve after initial equilibration between intra- and extra-vascular spaces (149).

RESULTS

Primary and secondary responses to POL. Although it has been observed

that *B. marinus* toads may have natural antibodies against *Salmonella* antigens with titers in the 1:0-40 range (141), no such antibodies against POL were observed in the sera of the animals used in this study. The capacity of marine toads to produce a secondary response has been investigated using different doses of POL (Fig. 24).

Toads immunized with 1.0 ng/animal had detectable antibodies one week later. This primary response peaked after 4 wks and declined after 8 weeks. A second injection of the same dose of antigen given 68 days after the first one induced a response with anamnestic characteristics. A new peak of antibody titer was reached within 3 weeks and was significantly higher than the primary one. Other characteristics of the secondary response observed in Mammals, i.e. longer duration of the response and increased production of 2-ME-resistant antibodies were absent in these toads.

The response to the higher dose of 100 ng of POL followed the same kinetics obtained with 1.0 ng, but the titer of the antibodies was higher at all times of assay.

A further increase in antigen dose to 10 μg induced a faster primary response which reached its peak at 5 wks and diminished after 8-9 wks. A second injection at this point stimulated a secondary response which failed to reach antibody titers higher than those obtained with 100 ng of POL. This response, however, lasted for more than 20 wks.

Humoral response to a high dose of POL. Toads injected with 100 μg of POL and kept at room temperature responded within 2-3 wks with very high levels of serum antibodies, reaching titers of 1:512,000 or more (Fig. 25). The response lasted for more than 34 wks and some toads still had significant titers of antibodies one year after a single immuniza-

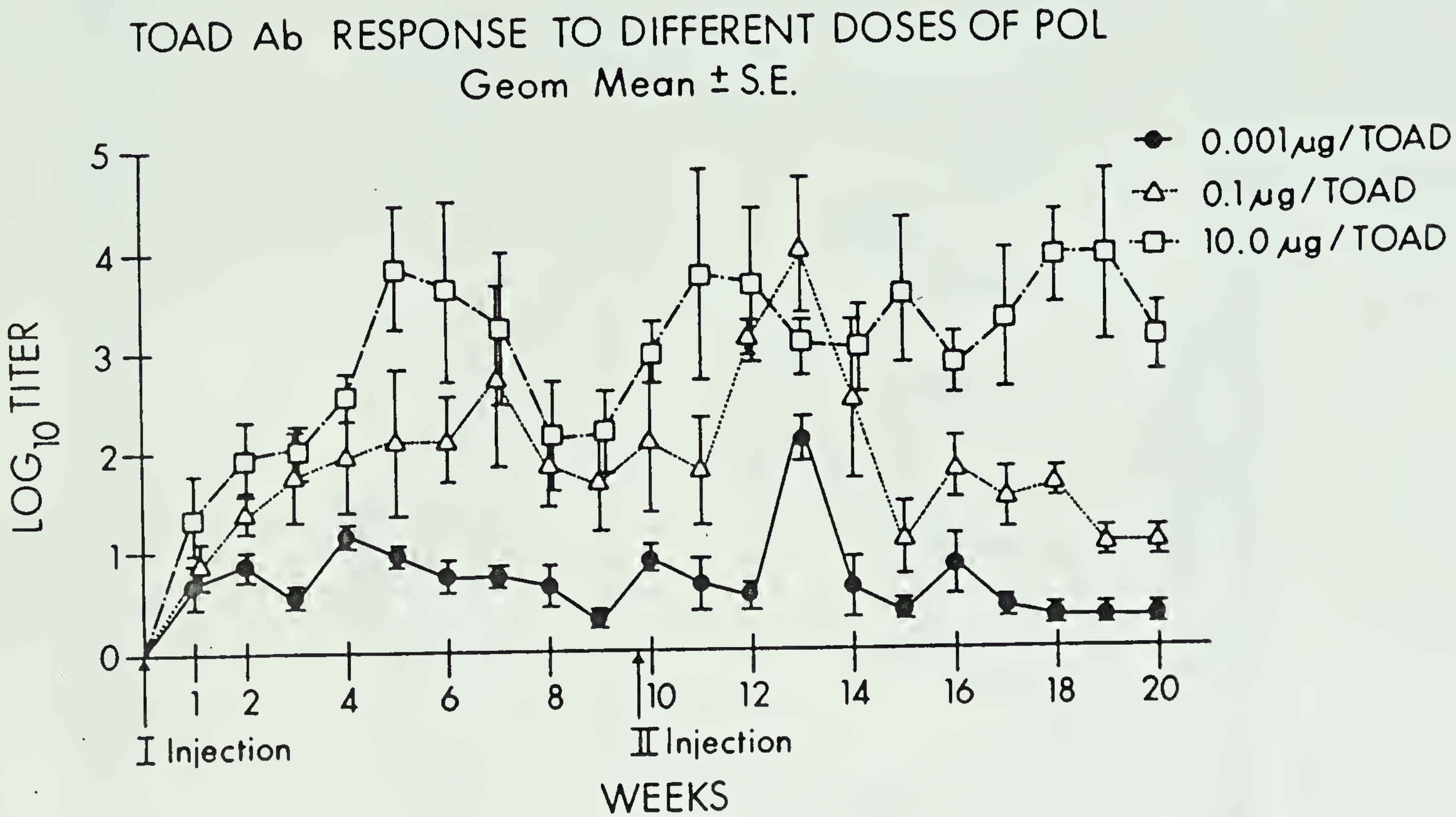

Fig. 24

Kinetics of serum antibodies in the primary and secondary responses of B. marinus to different doses of POL. Both injections consisted of the same dose of antigen. Each point represents the mean ± S.E. of the titers of 8 animals, 4 in each experiment repeated twice.

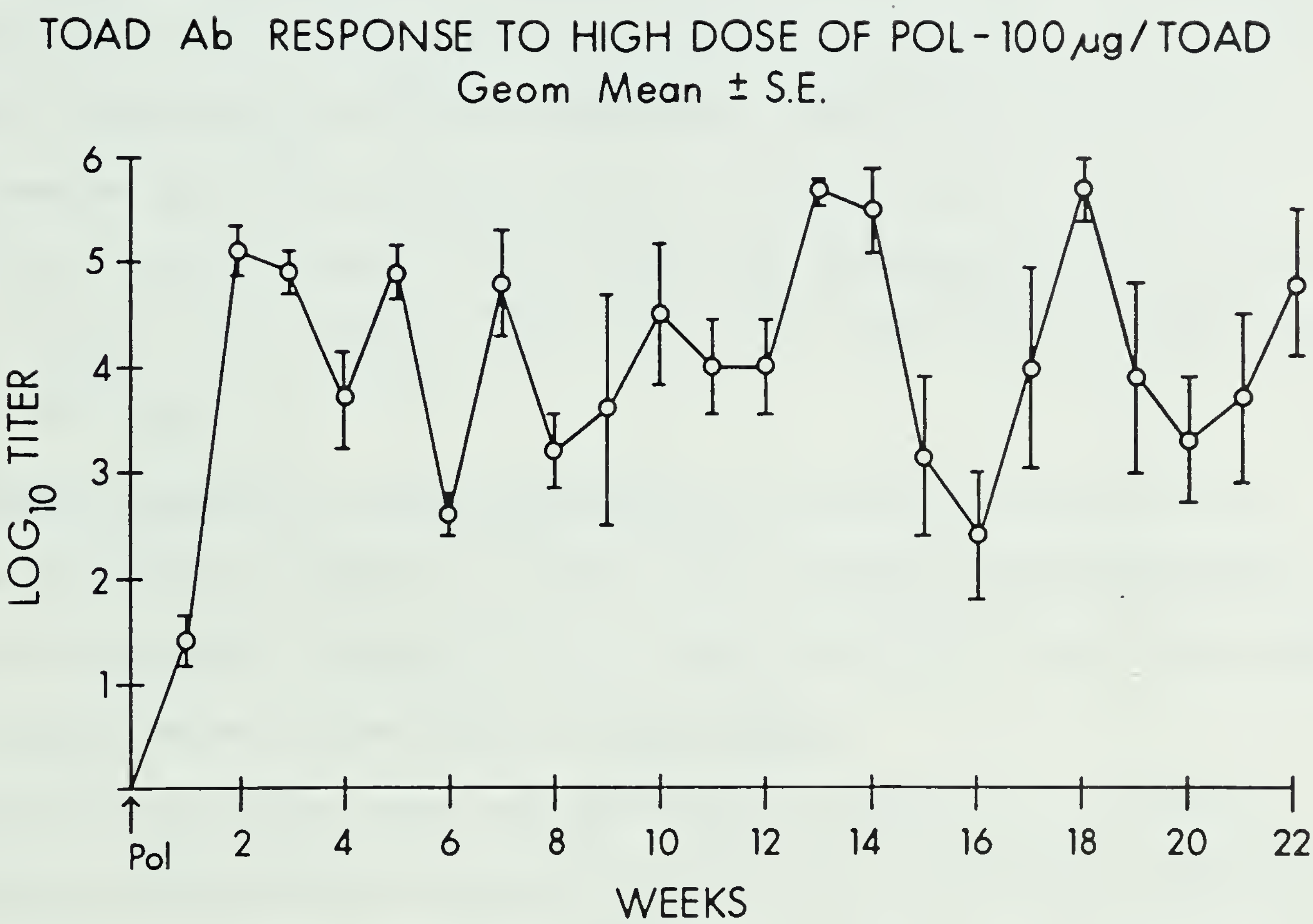

Fig. 25

Toad serum antibody response to 100 ug of POL. Each point is the mean $\pm$ S.E. of 10 animals from 3 experiments.

tion. This response though, rather than being constant, followed cyclic oscillations, in which peaks and troughs appeared at 2-3 wks. intervals, depending on the individual toad (Fig. 26).

Catabolism of immunoglobulins in toads. In order to find an explanation for the cyclic rise and fall of the antibody titers, the catabolic rate of immunoglobulins in the circulation was estimated. It was reasoned that such cycling may reflect some feedback mechanism by IgM antibody. For this purpose the IgM fraction of the serum proteins was isolated, labelled with ^{125}I and injected i.c. into normal toads. The analysis of the catabolic rate of N-IgM showed that these immunoglobulins have a half-life of 17 days in toads kept at 22^o, and 12 days in those kept at 37^o (Fig. 27).

Humoral response to sheep erythrocytes. Since SRC are considered to be antigens the response to which in mice requires the collaboration of at least two cell types (27), it was interesting to test the responsiveness of the marine toads to this antigen.

As shown in Fig.28, toads responded well to a dose of 2×10^8 SRC. Specific hemagglutinins appeared in the sera one week after immunization; their level peaked after 4 wks and declined after 9 wks. Subsequently only a large dose of SRC was capable of inducing a secondary response with a shorter lag-phase than that of the primary one; yet it failed to stimulate titers of antibody characteristic of an anamnestic response. Furthermore, the incubation of the anti-SRC sera at 57^o for 30 min, or with 2-ME, obliterated the antibody activity.

Suppression of the antibody response to POL by passive immune IgM. Different groups of 4 toads each were given I-IgM at doses of 6.4 mg or 0.64 mg/toad, I-IgG at a dose of 5.0 mg/toad and N-IgM at 6.0 mg/toad,

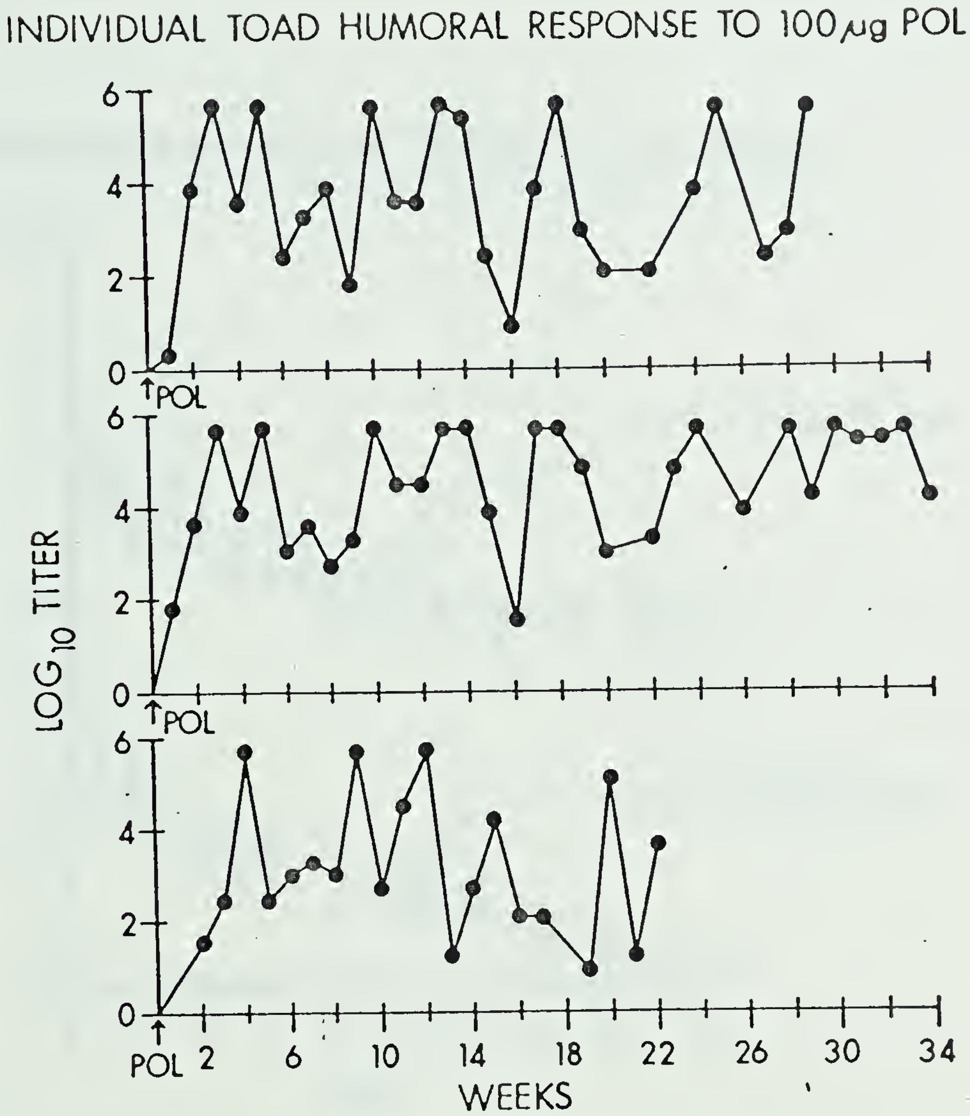

Fig. 26

Serum antibody response of 3 representative toads immunized with 100 µg of POL.

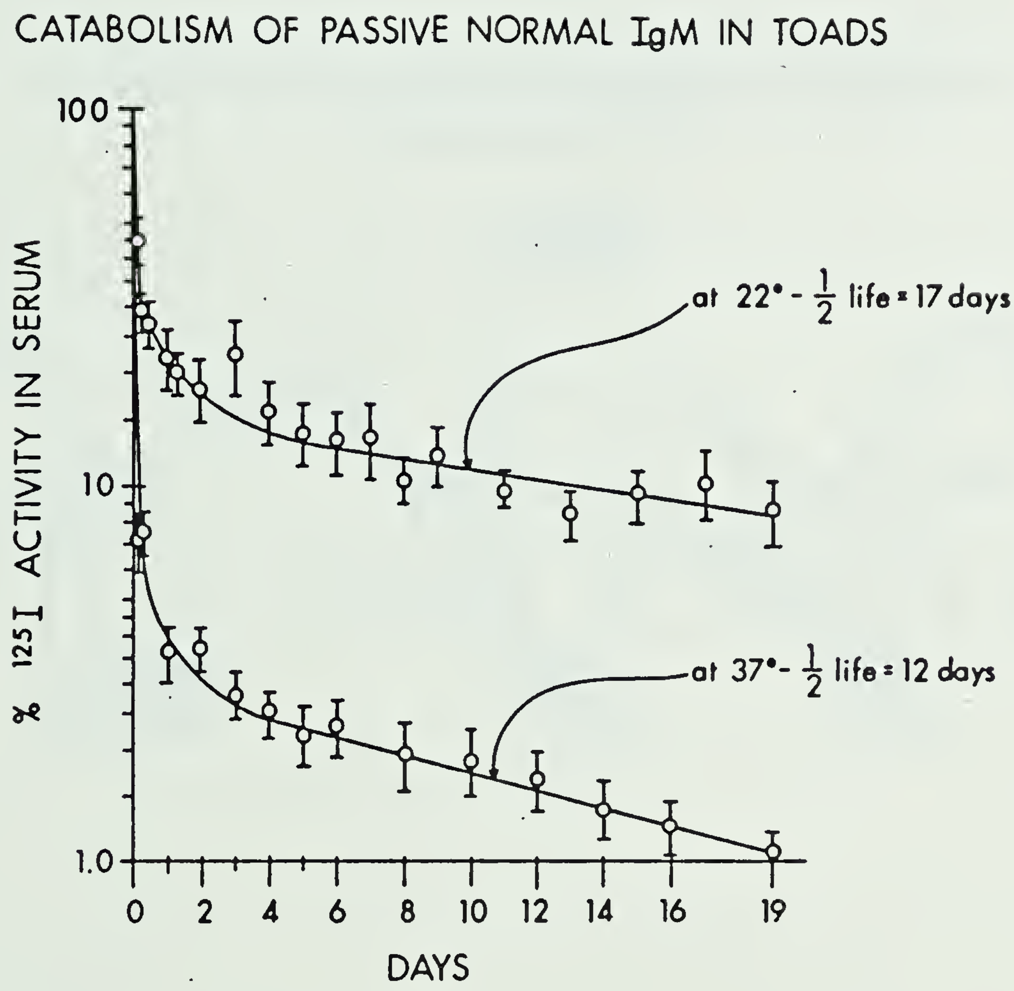

Fig. 27

Catabolism of passively administered normal IgM in toads. 4 animals, kept at each of the temperatures shown, were injected with 0.75 mg of ^{125}I-IgM. Each point is the mean $\pm$ S.D. of the cpm given by 50 ul of serum.

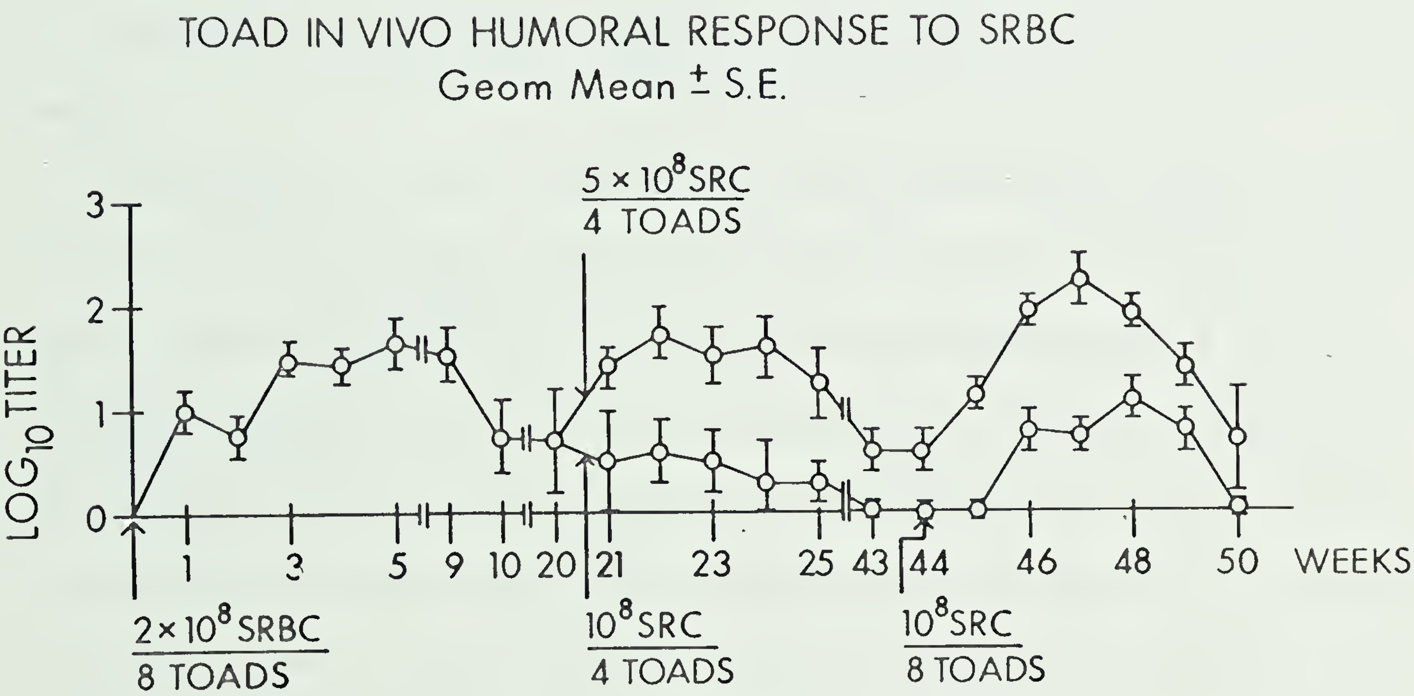

Fig. 28

Primary and secondary humoral response of toad against SRC.

three days after immunization with 100 μg of POL. All these proteins were administered i.p. in 1.0 ml of saline. The titer of I-IgM (6.4 mg/ml) was 1:32,000, that of I-IgG was 1:128 and both were sensitive to treatment with 2-ME. No anti-POL activity was present in N-IgM which was given for comparison, to test the effect of non-specific proteins on the antibody response. The normal level the serum proteins in unimmunized toads was 11.25 mg/ml and in immunized ones was 12.6 mg/ml.

The injection of I-IgG, N-IgM and diluted I-IgM had no effect on the humoral response to POL, which was similar to that of untreated immunized controls. In those toads injected with concentrated I-IgM the initial response appeared somewhat enhanced when compared with that of immunized controls (Fig. 29). Some clear difference between these two groups was however seen 70-80 days after immunization, in that the antibody titer was significantly lower ($p = 0.01$-0.05) in toads that had received a high dose of I-IgM.

DISCUSSION

In these experiments the antigen dosage has been proven to play an important part in the demonstration of a secondary response. The data presented here confirm the observations made in other species of Poikilotherms (5, 28, 146), but disagree with other reports which claimed a reduced immunological anamnesis in B. marinus (41, 57).

The response to a second dose of 0.1 μg or less of POL appeared to be anamnestic because of the shorter latent period and higher antibody titers than during the primary response. Another characteristic found in the secondary response of Homeotherms is the formation of mainly IgG

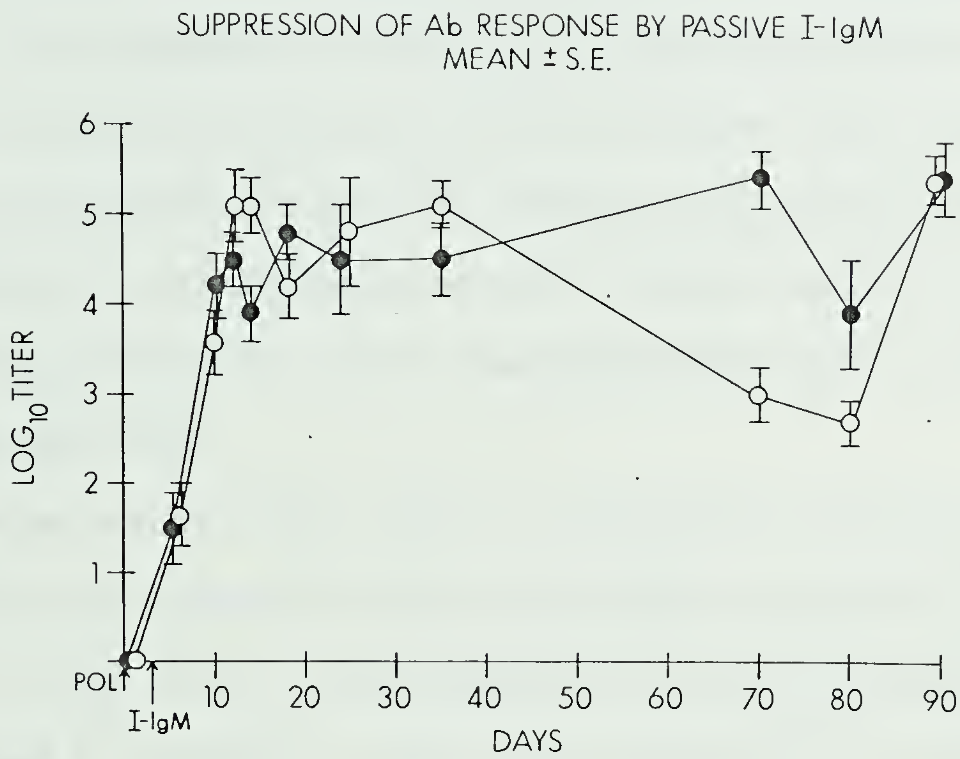

Fig. 29

Effect of passive IgM-antibody on the toad humoral response to 100 μg of POL. The antibody was injected i.p. 3 days after POL. Each point is the mean ± S.E. of 8 animals from 2 different experiments.

antibodies (164). These were not found in the marine toad, in agreement with the results reported by others who have used Salmonella H antigens (1, 43). The low anti-POL titer found after gel fractionation of immune sera in the fraction containing low-molecular-weight antibody (here called IgG for convenience) was probably due to contamination by IgM.

In toads immunized with T_2 bacteriophages some authors have found 17.9S and 7.3S antibodies in which the L chains appeared to be similar, but the H chains had different molecular weight, amino acid composition and carbohydrate content (1). Compared with human 19S and 7S immunoglobulins, toad IgM seems to have a similar migration pattern in gel electrophoresis, but the 7S immunoglobulins have a slower mobility than human IgG (43).

In Anurans the antigen seems to persist for various periods depending on the dose, especially when the animals are kept at temperatures below 25^{o}. It has in fact been shown that marine toads immunized with formalinized S. typhosa at 25^{o}, when transferred to a temperature of 35^{o} four months after the priming injection, produce a response higher than that of equally treated toads which received a booster dose of antigen (57). Persistence of the antigen, even though not demonstrated in the present study, seems to provide the most acceptable explanation for the long duration of the response to a single dose of 10 μg or more of POL. A cyclic appearence of AFC in mice after a single injection of poorly catabolized antigens (19, 133), has indeed been taken as evidence for a similar interpretation.

In toads kept at 22^{o} the half-life of IgM is 17 days which closely matches the periodicity of the peak titers observed in the experiments

described here. In marine toads equally immunized but kept at 37^{o} the cycling phenomenon has not been observed by others (41), probably because of faster processing of the antigen and of the shorter half-life of the serum proteins at that temperature.

The dose-response kinetics observed in toads immunized with POL correlate with the curves obtained in the response of rats to the same antigen, with the only difference that the immunogenic dose-threshold seems to be lower in toads (0.001 μg) than in rats (0.1 ug) (118).

In contrast to the experiments with POL, toads injected with SRC failed to demonstrate a truly anamnestic response. This disagrees with the finding that the larvae of <u>A. obstetricans</u> can respond to a second immunization with SRC with both a shorter lag-phase and higher antibody titers than in the primary response (52).

Passively administered IgM antibody, depending on its concentration, can have an enhancing effect on the IgM response of mice. In contrast, 7s antibody has been shown to have the opposite effect by suppressing the response (83). The injection of 19S antibody seems to have the same effect in toads, at least as far as the early response is concerned. The significant suppression by IgM antibody of the late response to POL is in agreement with the observation that passive 19S antibody can prevent the cyclic appearance of antibodies against <u>E. coli</u> endotoxin in mice (19).

CHAPTER V - IN VITRO IMMUNE RESPONSE OF TOAD SPLEEN CELLS TO POL

MATERIALS AND METHODS

Animals. B. marinus toads were acquired and kept at room temperature as described in Chapt. III.

Antigen and Immunization. POL from S. adelaide strain SW 1338, prepared as described in Chapt. II, was used throughout these experiments. As commented further in the text, some toads were injected i.p. with 100 μg of POL each.

Culture medium. Eagle's Minimal Essential Medium (MEM) (GIBCO) was diluted in double-distilled water and supplemented with FCS. Isotonic medium was made of MEM, H_2O and FCS in the ratio 58.5%:31.5%:10%. The osmolarity of this medium was measured in a vapor pressure osmometer as described in Chapt. III. To prevent infection 100 u. of penicillin and 100 μg of streptomycin (GIBCO) and 250 u. of mycostatin (Nystatin, Squibb, N.Y.) were added per ml of medium.

Preparation of cell suspensions. Single-cell suspensions were prepared from one or more spleens as described in Chapt. III, in culture medium on ice. The cells were washed 3x in medium, spun at 330 g for 10 min, counted in a hemocytometer and tested for viability by eosin dye exclusion (75).

Culture conditions. 20 x 10^6 cells in 1.0 ml of medium were grown on dialysis membranes which closed the bottom-end of a glass tube and were tightly sealed with a ring of Silastic tubing (Dow Corning); the glass tubes were inserted into Erlenmeyer flasks containing 50 ml of medium (42, 101). In successive experiments the culture flasks were reduced in size to contain 6 x 10^6 cells in 0.3 ml of medium. Unless otherwise stated, all the results refer to this latter cell concentration per

culture. The cells were incubated at 37° in a humidified atmosphere adjusted to 10% CO_2.

For each experimental point the cultures were prepared in triplicate and each experiment was repeated at least three times.

Harvesting. At appropriate times the cells were resuspended from the dialysis membrane on which they had sedimented. The suspension was removed with a pasteur pipette and transferred into a 5 ml glass tube in ice. The membrane was washed once with medium to pick-up left-over cells. After centrifugation at 330 g for 10 min the cells were washed 3x in L-15 and the AFC counted by the bacteria-adherence colony assay (39). The results are expressed in terms of mean numbers ± S.E. of AFC/culture.

Determination of ^{3}H-Thymidine incorporation. Seven hrs before harvesting cultures of 6 x 10^6 cells were transferred into sterile glass tubes and incubated with 2 uCi of ^{3}H-Thymidine (N.E.N., 2.0 Ci/mM specific activity). The cells were harvested according to the following procedure (10): culture tubes were chilled at 4° and then centrifuged at 650 g for 10 min. The supernatant was discarded and 5 ml of cold 5% TCA were added. After 30 min at 4° and following centrifugation, the precipitate was resuspended in 0.8 ml of cold NaOH (0.1M), followed by the addition of 4.2 ml of 6.7% TCA, and stored at 4° for 30 min. Then the precipitate was spun down and the pellet was dissolved in two drops of 10% tetraethylammonium hydroxide (TEAH) (Eastman Kodak Co., Rochester, N.Y.). The solution was diluted in 2.5 ml of scintillation fluid and poured into a scintillation vial (Packard) already containing 7.5 ml of the same fluid. This was made of the following cocktail (10): ethanol (absol.) 280 ml, xylene 468 ml, 1,4-dioxane 468 ml, naphthalene 98.4 gr, PPO (2,5-diphenyloxazole)(Amersham/Searle, Arlington Heights,

Ill.) 6.13 gr, alpha-NPO (2-(1-naphtyl)-5-phenyloxazole)(Nuclear, Chicago, Ill.) 0.0613 gr.

The vials were counted in a Packard liquid scintillation counter. The results are expressed as a ratio between the counts per minute (cpm) given by antigen-stimulated cultures and the cpm of control cultures. The standard deviations were calculated from the values obtained from at least 6 replicate cultures.

Staining of tissues in culture. In order to observe the morphology of the cells in culture the dialysis membrane on which they had settled was cut from the inner tube of the culture flask and placed in a petridish, taking care to avoid any agitation which could have upset their arrangment. The dish was slowly flooded with Bouin's fixative, left for 24 hrs at room temperature and then the tissues were processed for staining with methylgreen pyronin (123a).

RESULTS

Antibody-forming cells in vitro after immunization in vivo. The quality of the culture conditions was tested by growing, in the absence of antigen, spleen cells from toads immunized *in vivo* for 2 days. By this method it was possible to follow both cell viability and immune-responsiveness in culture conditions which were new for toad cells, without uncertainty about the effective activation of the cells by the antigen. For this experiment, each culture-flask was inoculated with 20×10^6 cells from 2-3 pooled spleens. Antibody-forming cells were observed after 4 days *in vitro*; their number was highest one day later and declined thereafter (Fig. 30). The cell viability was 85% after 4 days, stabilized around 50-60% between days 5 and 7, and dropped to 30% after 9 d.

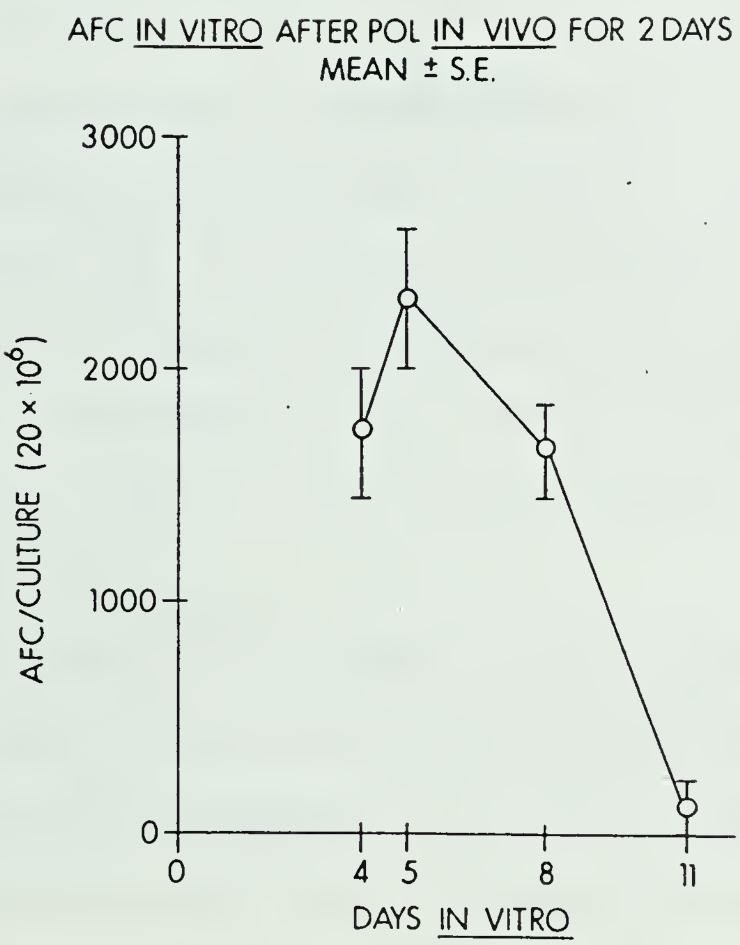

Fig. 30

Toad AFC response <u>in vitro</u> after immunization in vivo with 100 ug of POL. Each point is the mean $\pm$ S.E. of 3 experiments with triplicate cultures in which large flasks with 20×10^6 cells were used.

Immunization and response in vitro. Cultures of 20 x 10^6 cells from pooled spleens of normal toads were incubated with different concentrations of POL in the range between 0.01 and 10 ug/ml. A congruous number of AFC was observed after 5 days, but the peak response of 1945 ± 273 AFC/culture was obtained at day 6 with the antigen concentration of 1.0 µg/ml. Concentrations of POL of 0.01 and 10 ug/ml gave respectively 35% and 30% of the response induced by the optimal dose of 1.0 µg/ml.

In this culture system the cells sedimented on the dialysis membrane forming four strata which mostly included, starting from the bottom, erythrocytes, large leukocytes, small leukocytes and damaged cells (Fig. 31). Fig. 32 shows an enlargement of a portion of the previous picture to evidentiate the large number of blast cells present in the culture after 5 days. At this time pyroninophilic cells were already present (Fig. 33).

Toad AFC looked similar to those of mice (39) and varied in size, but most of them were medium (Fig. 34) and large (Fig. 35) lymphocytes.

Effect of cell concentration. The results obtained in the large flasks from pooled spleen cells were inconstant and in some experiments very few AFC were found. Suspecting immune inhibition by histoincompatibility reactions, it was decided to reduce the dimensions of the tissue-culture model, so that the number of cells from a single spleen would be sufficient for each experiment. To assess the effect of cell concentration small flasks were inoculated with different numbers of cells. As shown in Fig. 36, at day 6 the response increased proportionally with the cell concentration; when this was higher than 6 x 10^6 cells/culture, reduced responses were obtained, probably due to overcrowding. Therefore the optimal number of 6 x 10^6 cells/culture was adopted for

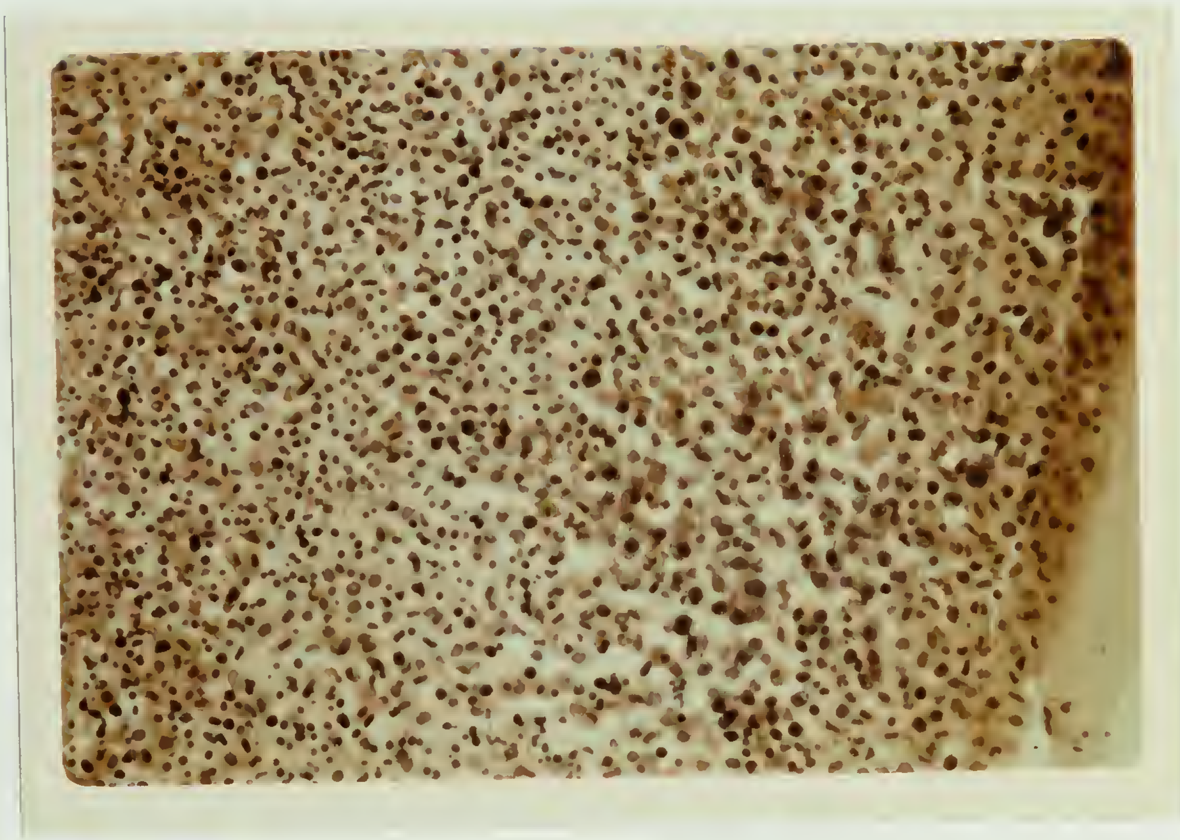

Fig. 31

Histology of toad cells after 5 days in culture. The vertical section shows the cell arrangement. The dialysis membrane is on the right. Methyl-green pyronin. x 400.

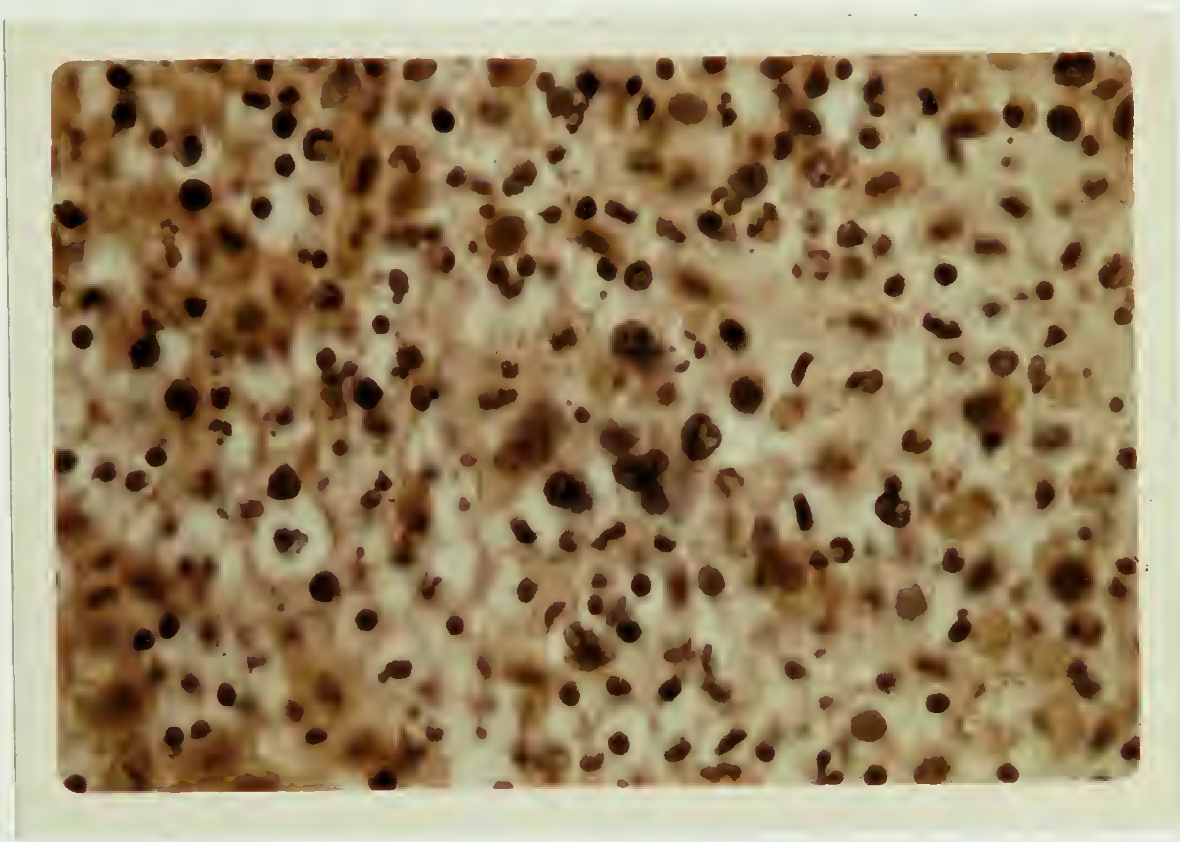

Fig. 32

Histology of toad cells after 5 days in culture, as in Fig. 5-2. x 800.

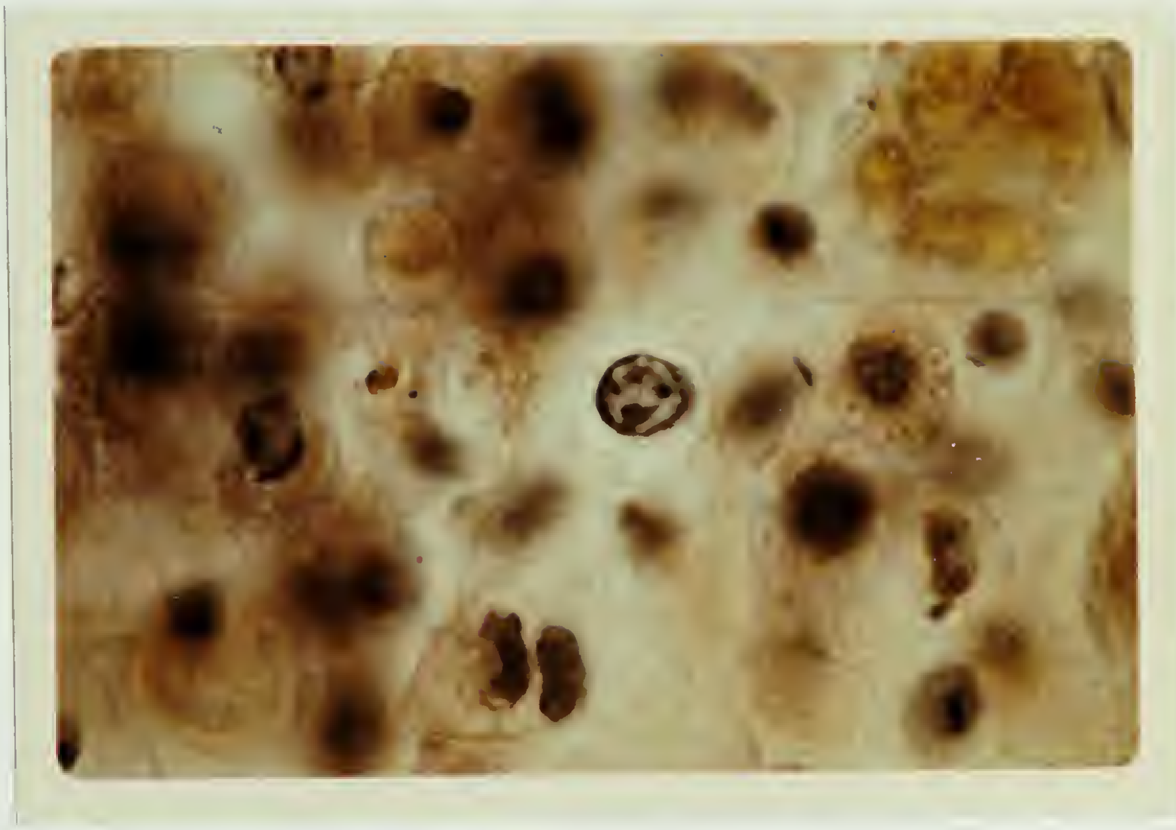

Fig. 33

Pyroninophilic cell (in the center) in 5 days culture of toad cells. Methyl-green pyronin. x 1600.

Fig. 34

Medium-size antibody-forming cell from day 6 culture. The antibodies cause the adherence of Salmonella bacteria. Wet mount, therefore the bacteria move and appear fuzzy in this photomicrograph. x 2400.

Fig. 35

Large-size AFC from day 6 culture. This picture was taken at higher speed to better evidentiate the adhering bacteria. x 1200

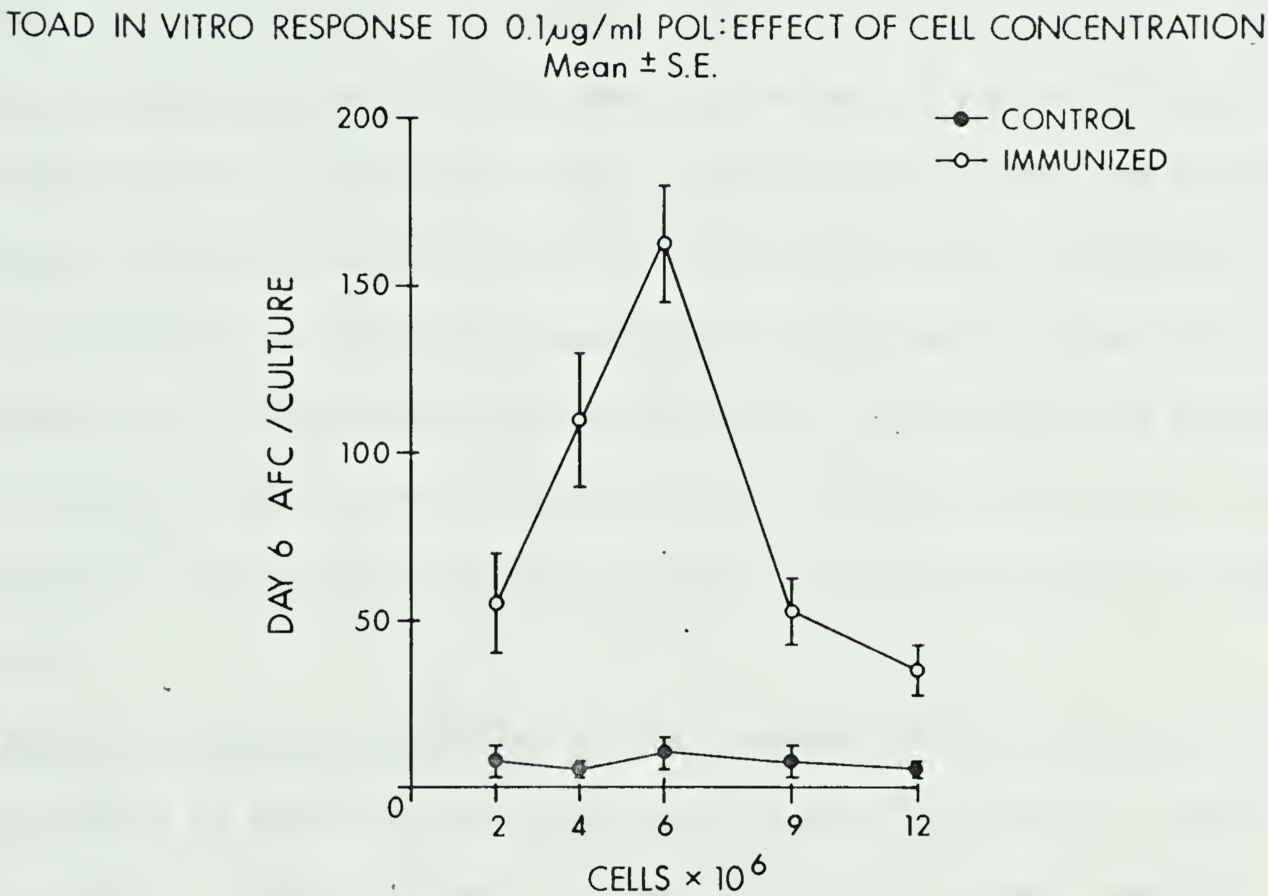

Fig. 36

Effect of cell concentration on the in vitro AFC response of toad spleen cells to 0.1 μg of POL. The cultures were grown in 25 ml flasks and assayed at day 6. Each point is the mean ± S.E. of 9 triplicate cultures from 3 experiments.

the subsequent experiments.

Effect of the antigen dose. As shown in Fig. 37, the optimal dose of POL was 1.0 µg/ml of cell suspension. A dose of 0.1 ug/ml has been found to induce a response not significantly lower than 1.0 ug/ml. Doses of 0.01 and 10 µg/ml produced smaller numbers of AFC. with kinetics distinctly different from that induced by the optimal dose.A small number of AFC was observed at day 5 in control cultures incubated without antigen.

Effect of different sera. Since there is evidence that the presence of allogeneic or xenogeneic serum can differently affect the response *in vitro* to PHA and in MLC (69), this aspect was also investigated, even though not in detail. Cultures of 20 x 10^6 pooled spleen cells produced 165 ± 15 AFC/culture when grown in medium with 10% normal toad serum and 1837 ± 146 in the presence of 10% FCS. Therefore the presence of toad serum suppressed 90% of the AFC seen in the presence of FCS.

The uptake of tritiated Thymidine. The response to POL *in vitro* was also studied in terms of cell proliferation and ^{3}H-Thymidine uptake. The results of this experiment, shown in Fig. 38, suggest that DNA replication precedes the AFC response by 24-36 hrs. At the point of maximal incorporation of ^{3}H-Thymidine the cpm values in the cultures incubated with the antigen were 4.15 times higher than in the control ones, (mean values ± S.D.: control 4164 ± 1014; stimulated 17886 ± 5903).

Persistence of the response in vitro. The decline in the response to various doses of POL, observed after 6 days, was somehow in contrast with the kinetics observed *in vivo*. On the other hand, as mentioned above, the cell viability *in vitro* declined sensibly after 7 days.

To investigate the long-term functionality of the cells in culture, at

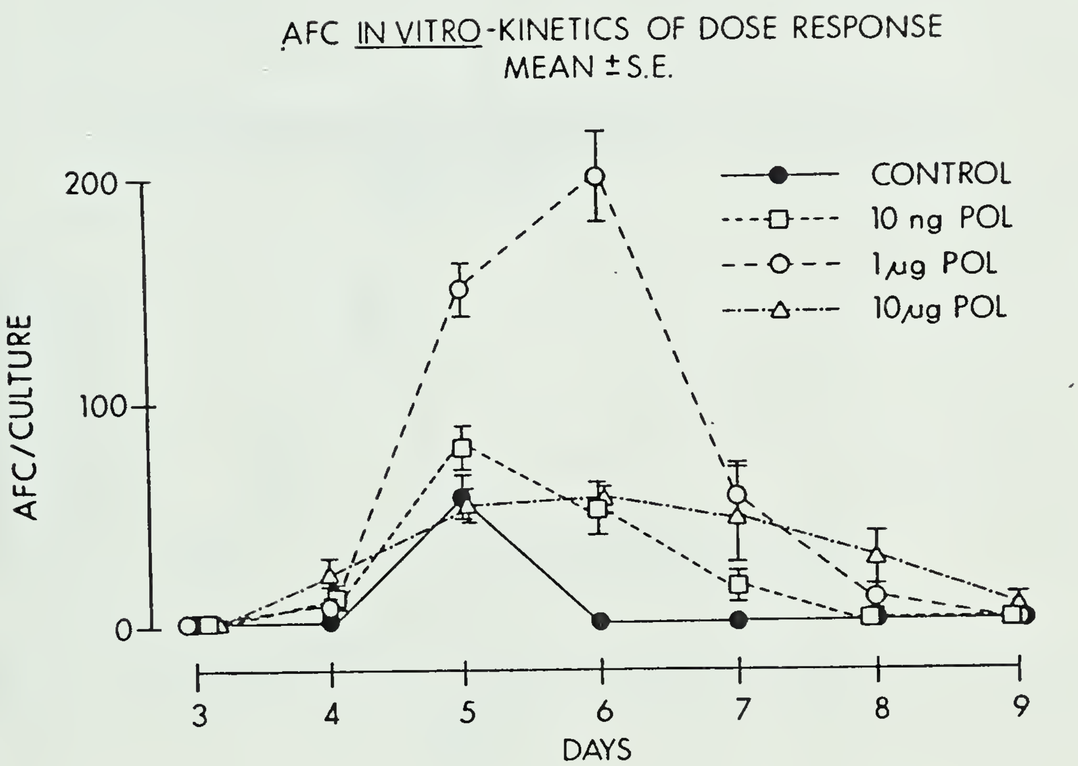

Fig. 37

Kinetics of the toad spleen AFC response *in vitro* to different doses of POL. Cultures of 6 x 10^6 were grown in 25 ml flasks. Each point is the mean ± S.E. of 12 replicate cultures from 4 experiments.

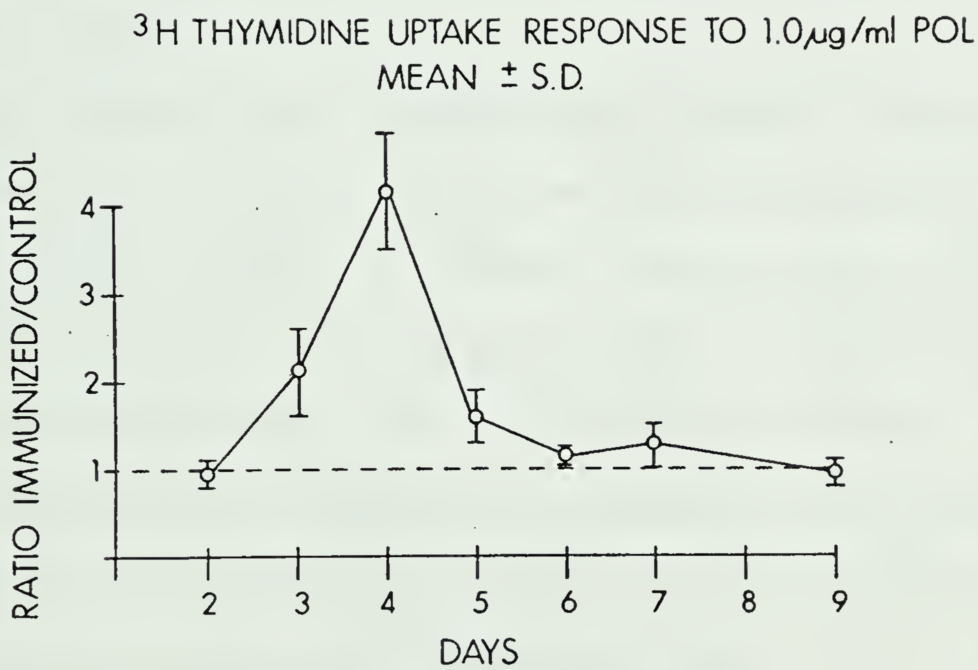

Fig. 38

^{3}H-thymidine uptake response of toad spleen cells _in vitro_. Cultures of 6 x 10^6 cells were incubated with 1.0 ug/ml of POL. The ratio on the ordinate was obtained by dividing the cpm of 6 replicate cultures incubated with POL by the cpm of 6 cultures without antigen. The data have been pooled from 2 experiments.

7 days the medium in the reservoir was replaced with a freshly-prepared batch, and the cultures were tested later for AFC and cell viability. Although the viability did not rise above 40%, the number of AFC increased again 12 days after immunization and reached a new peak at day 15 (Fig. 39).

DISCUSSION

The results reported in this chapter demonstrate that a primary immune response can be induced in single-cell suspensions of amphibian spleen *in vitro*.

It had been previously reported that fragments of *X.laevis* spleen could respond to SRC (9) and cell suspensions of *X. laevis* and of *B. marinus* could react to PHA and to allogeneic cells *in vitro* (69, 163).

The immune response has been analysed by two parameters, the increase in number of AFC and the rate of ^{3}H-Thymidine uptake. On the assumption that AFC result from division of precursors cells, those two functions appear to be correlated with each other, the kinetics of thymidine incorporation preceding the appearance of AFC.

The kinetics of the *in vitro* response appear different from those observed *in vivo* under the same conditions of antigen and temperature (43). In comparison, the *in vitro* response not only produced less AFC, but also its peak response occurred one day earlier and its lag-phase and duration were shorter. The rapid decline after 6 days of culture did not seem to be due to cell death or loss of the antigen, but rather to nutritional factors, since the response could be restored 5 days after the replacement of the medium.

The differences between *in vivo* and *in vitro* responses of toad spleen

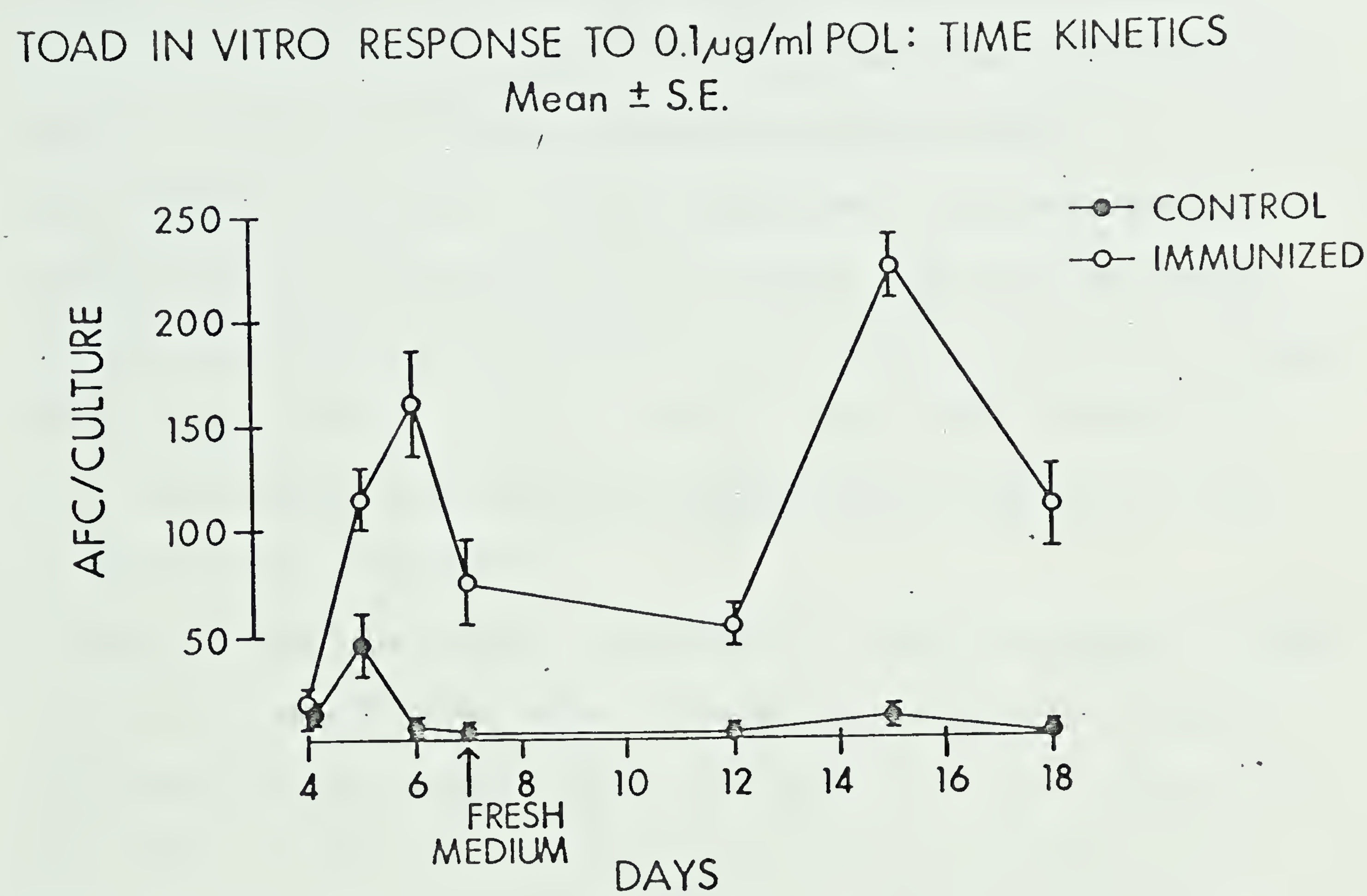

Fig. 39

Long term <u>in vitro</u> AFC response of toad spleen cells, when the medium is changed after 7 days. Cultures of 6 x 10^6 spleen cells were incubated in small flasks. Each point represents the mean $\pm$ S.E. of 6 replicate cultures from 2 experiments.

cells are in agreement with similar effects observed in cultures of mouse cells (42). Yet some variations seem to exist between the responses of mouse and toad spleen cells *in vitro*.

The peak response was seen in mouse cells at day 4, while in toad cells it occurred at day 6. This difference agrees with the results reported in Chapt.III, where it appeared that toad ABC undergo blastogenesis 3 days after immunization, while in mouse spleens this process may already be observed 24 hrs after antigenic stimulation (93).

More antigen is required to induce immunity and unresponsiveness in cultures of toad cells than in those of mouse. Therefore the possibility that toad cells are less effective in handling the antigen *in vitro* has to be considered. In fact it has been shown that fresh SRC have to be added weekly to cultures of *X. laevis* spleen fragments in order to obtain a good response (9).

Cultures of toad spleen cells yielded AFC in numbers comparable to those found in cultures of mouse cells. In both systems there is a logarithmic increase of AFC between cultures containing 5-6 x 10^6 and 20 x 10^6 cells. This increase cannot be entirely attributed to lack of close cell contact at low concentration of cells, as proposed by others (42), since as few as 2 x 10^6 toad cells produce a response which is just proportionally lower than the one obtained with larger concentrations. The logarithmic increase seems more likely due to differences in the number of AFC-precursors which, being less numerous when the total number of cells in culture is lower, give rise to fewer effector cells.

The decrease in number of AFC, observed when the cell concentration was above the optimal one, has also been found in cultures of mouse cells (42); it could be the consequence of cell crowding, thereby lea-

ding to the accumulation of toxic products.

The use of cultures with large numbers of *B. marinus* spleen cells does not seem appropriate, due to the large variability of the results. Since these toads bred randomly, and their spleen cells respond very well in MLC (69), the suppression of the humoral response because of an allogeneic effect (86) is potentially always possible.

The nature of the background of bacteria-adherence colonies present in control cultures at day 5 has not been investigated. However it could be due to some non-specific mitogens present in the culture medium, as also shown in other experiments involving PHA and MLC stimulation *in vitro* of *B. marinus* spleen cells (69).

CHAPTER VI - GENERAL DISCUSSION AND CONCLUSIONS

GENERAL DISCUSSION

The goldfish and the marine toad have afforded the opportunity to investigate some as yet unexplored aspects of the humoral immune response in lower Vertebrates.

In both species immuno-induction and antibody response could be studied at the cellular and humoral level at different temperatures, antigen dosages and durations.

A characteristic unique to the immune system is the specificity of the reaction to a given antigen. This specificity has been demonstrated to qualify the afferent branch of the immune system, in terms of recognition of the antigen, as well as the efferent one which results in the production of specific immunoglobulins (117).

In Homeotherms the surface kinetics following antigen-binding seems to depend on the biological activity of the cell and do not occur at 0^{o} (44). Experiments on the temperature-dependence of cap formation in mouse cells (Fig. 7, Table IV) agree with the hypothesis that at this temperature the phospholipid bilayer of the membrane is in a gel state (90). Few experiments conducted in hibernating Mammals have shown that immune responsiveness is lost during hibernation when the body temperature drops from 37^{o} to 4-6^{o} (6). The finding that in these animals kept at room temperature the intensity of the primary immune response reached a minimum in January has suggested that unresponsiveness in hibernation can be due to a primary circennial depression of immunocompetence (145). Nevertheless secondary factors like suppression by brown fat, demonstrated *in vitro* (144), and rigidity of the membrane

of immunocompetent cells could also contribute to the inhibition of the immune response under natural conditions.

In contrast, poikilothermic animals can mount an immune response at temperatures below 10^{o} (130, 131 and the present work).

At the cellular level the biological activity seems to be maintained even at 0^{o}, as it appears from our experiments on antigen-binding and cap formation. This latter reaction has not been studied in detail in the marine toad, but numerous cap-forming cells have been seen throughout the cell-separation experiments reported in Chapt. III. These observations correlate with the previous finding that the membrane phospholipids of Poikilotherms contain more unsaturated fatty acids the lower the temperature to which they are adapted (66, 151), thus mantaining their fluidity. This phenomenon supports the fluid mosaic model of the cell membrane, according to which certain membrane-intrinsic proteins, such as the immunoglobulins, are monomolecularly dispersed in the phospholipid matrix, and binding of a ligand would thermodynamically favor the aggregation of the protein-ligand complexes (147). While the experiments on "suicide" (3), blastogenesis (93 and the present work) and transfer of an adaptive secondary response (84) by ABC have provided concurring evidence that these cells are immunocompetent and precursors of AFC, the significance of cap formation after antigen-receptor interaction remain unclear. Since it has been shown that Concavalin-A can inhibit antigen-induced capping but not the in vitro immune response of cells from mice deprived of T-cells (96), cap formation does not appear to be an essential step in immune induction.

It is noteworthy that this phenomenon is present throughout the whole vertebrate phylum and therefore could be a basic regulatory mechanism

to remove from the cell surface the antigen which could otherwise accumulate and reach tolerance-inducing levels (46), or interfere with other cellular functions.

The acclimatization of goldfish at 4^{o} seems to have put in evidence the effect of temperature on the regulation of cellular functions beyond the immune response. The number of cells in the spleen and that of receptors on the ABC are both lower in cold-acclimated than in warm-acclimated fish. These differences are expectable and seem to reflect physiological adaptations to severe environmental conditions. The fact that a similar difference was not significantly expressed in the humoral immune response is probably due to the large variability in responsiveness observed in many species of Poikilotherms, especially when they are outbred (53), and this complicated the statistical analysis of the results.

The events occurring in Poikilotherms between immunization and the first appearance of AFC had not been investigated previously. The availability of a tritium-labelled antigen, which under the light microscope allows high enough resolution for an accurate localization of the antigen on the cells, has permitted the study at the cellular level of the early processes of immune induction, before antibody production was started. In mice the injection of POL induces blastogenesis of the ABC within 24 hrs (93), and these cells can initiate a primary immune response <u>in vitro</u> (45). In goldfish and marine toads this antigen seems to induce a similar response, although with slower kinetics than in the mouse. Cell proliferation was evident after 2 days in the fish and after 3 days in the toad. Since both animals were kept at 22^{o}, the delay in the response could be attributed to the temperature, which is lower than the

body temperature of the mouse. Yet this does not seem a satisfactory explanation, at least as much as the toad is concerned. In fact AFC can be found in the mouse 2 days after immunization (136), whereas they are not detectable before 3 days in toads kept at 37^{o} (91). Furthermore, the in vitro AFC response takes 6 days to peak for toad cells, but only 4 days for mouse cells (42), under the same conditions of temperature and culture system. The delay in immune induction observed in the two species studied here seems to be an expression of their phylogenetic difference from the mouse.

The goldfish is the most primitive of the three species discussed. The wide span of sedimentation velocities of ABC in normal fish suggests that these cells do not form a uniform population of specialized cells, as in toads or mice, but may represent different stages of differentiation from the original stem cells.

The ABC found in the spleen of the marine toad seem to reproduce the intermediate stage of evolution of this animal. Their size distribution tends to coincide with that of the ABC found in mouse spleen (93) but, as suggested by others (91), they seem to follow a different pathway of differentiation into AFC, involving a series of halving divisions without intermediate growth.

Our data suggest also that ABC migrate from the spleen 1 and 2 days after antigenic stimulation. This latter characteristic seems common to both fish and toads and probably means that in these two species the development of AFC is not a closed system limited to the spleen, but involves also the blood. In fact high numbers of AFC are found in the blood of toads, where they appear even sooner than in the spleen (91), while it is well known that these cells are almost absent in the blood

of Mammals. Both fish and toads lack a good system of peripheral lymph nodes, and their blood seems to compensate for this deficiency.

One of the aims of the present study was to bring some clarity in the controversy about the presence of immune anamnesis in Amphibia (53). The capacity of marine toads to produce a secondary response, as well as the regulatory effect on it of the antigen dose, seems to have been demonstrated here. The results obtained with doses of POL below 0.1 μg are in agreement with those observed in the margate Haemilon album (28) and in the lizard Ophisaurus apodus (5), in the sense that a second injection of the same dose of antigen given when the primary response has declined induces faster and higher titers of antibody than the priming immunization. In contrast, repeated injections of SRC seem to induce a response which is faster but not stronger than the primary one. Since similar results have been obtained in the midwife toad Alythes obstetricans, it appears that the amphibians have some deficiency in the response to cellular antigens, perhaps due to the fact that in these animals the thymus involutes after metamorphosis (53), a conclusion which at present is totally speculative.

A characteristic previously not observed in Poikilotherms is the cyclic response of B. marinus to the injection of a high dose of POL. In those Mammals in which this type of response has been observed it has been attributed to the persistence of the antigen (19, 133). This seems to be also the case in toads, as it is suggested by the in vitro experiment in which a second peak of AFC was obtained when the medium in the cultures was renewed without further addition of antigen (Fig. 39).

A difference between the toad and Mammals could be found in the longer period of the cyclic fluctuation of the response in this amphibian. As

mentioned above, this poikilotherm seems to have an immune response slower than in Mammals, both *in vivo* and *in vitro*. This difference was also demonstrated by the slow catabolic rate of passively administered immunoglobulins, which in toads have a half-life of 12 days at 37^{o}, while in Mammals it oscillates between 4 days in the mouse and 6 days in the rabbit (149).

CONCLUSIONS

This study of the different phases of the humoral immune response in two species of Poikilotherms has shown that a basic pattern characterizes the response and appears to be common to all Vertebrates. In addition, Poikilotherms have the capacity to respond immunologically under a wide range of temperatures, and their reactivity is improved by previous, gradual acclimatization to the wanted temperature.

The acclimatization of goldfish at 4^{o} has allowed the demonstration of immune responsiveness at that temperature, at which hibernating Mammals do not respond.

After antigen-receptor sites interaction (and cap formation) the immuno-competent cells of both goldfish and toads undergo proliferation and migrate from the spleen, probably into the circulatory system.

In vivo studies of the primary and secondary response in marine toads have shown that both the antigens used, POL and SRC, are good immunogens, even though POL appears preferable because it induces better primary and secondary responses. In the toad the secondary response can be demonstrated by modulating the antigen dose, since large doses tend to induce a high response which cannot be further stimulated in a secondary fashion. In this animal the secondary response is characterized

by the faster appearence of higher titers of antibody, but not by the secretion of 2-ME-resistant immunoglobulins. The cyclic appearance of peak-titers of antibody after a single high dose of POL seems to depend, among other factors, on the rate of catabolization of the circulating immunoglobulins, which is slower than in Mammals. In both fish and toads the immune response is characterized by kinetics which are slower than in higher Vertebrates.

A new approach to the investigation of immune reactivity in Amphibians has been initiated by the induction of a primary immune response _in vitro_ in suspensions of dispersed spleen cells using a soluble protein antigen.

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